

# Stem Cells

From Basic Research to Therapy

Volume 1

**Basic Stem Cell Biology,  
Tissue Formation during Development,  
and Model Organisms**

*Editors*

**Federico Calegari**

**Claudia Waskow**



**CRC Press**

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*Editors*

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# Preface

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## The Stem Cell Revolution

We occasionally witness fundamental changes altering the way in which we perceive the world. These sudden changes, sometimes called *revolutions*, may influence very diverse aspects of our every-day-life from politics to health care, from telecommunications to finance. Following a seemingly irreversible historical trend, changes are becoming more and more frequent, to the point that we start to get used to them and barely pay any attention anymore. Not because of that, these *revolutions* are any less important.

Stem cells are perhaps the last of a series of *revolutions* in biomedical sciences that slowly build up over time to explode in the last decade. Stem cells are, and will continue to be for years to come, a source of spectacular scientific achievements and shameful frauds, gold mine for pharmaceutical corporations and ruin for others, a charming hope to unfortunate patients and abhorrence to entire political administrations. Stem cells are the reason why an abstruse terminology limited to a few experts moved from the labs to the streets to be debated, nearly on daily basis, on worldwide media. Examples include *animal cloning*, *gene therapy*, *tissue-replacement*, *regenerative therapies* and so forth.

On a historical perspective, it is hard to find a subject in biology that has arisen more controversial feelings and debates at all levels of society. Perhaps the dispute over the risks of gene recombination in the '70 leading to the Asilomar Conference comes to mind as a worthy competitor but to find a clear winner we need to go back by over a century to consider the theory of evolution by Darwin and Wallace. In essence, we are witnessing here a quite remarkable *revolution*, and we should pay attention to it. To do so we first need to understand what stem cells are, which led us to consider the possibility of editing *Stem Cells*.

As it should be expected, a remarkably high number of books about stem cells are already available, in all forms and formats. As we checked the count was over 13,000; and the number is rising as a true *revolution* deserves. However, we did not need to read them all to realize that the overwhelming majority of these books are extremely specialized and of

a rather narrow spectrum. Hypothetical examples in this context may range from “*Stem cells in the gastrointestinal tract*” to “*Stem cell policies in central Africa*”. Nothing bad about specialised book, we need them too, but our ambition with *Stem Cells* was to provide the reader with a solid overview about stem cells in all the relevant contexts; all in *one* book. We soon realized that the relevant contexts were very many and that a fair coverage would need to include stem cells in the most relevant tissues, in species as diverse as plants to human, during development, adulthood and disease, and in specific applications ranging from therapy to commercial exploitation, each with its specific legislations and ethical considerations; all in *one* book. The challenge was daunting and as we write this preface, we are still surprised that we did not give up our project at that point... yet the challenge motivated us and perhaps the fact that we ourselves are scientists working on basic stem cell research gave us the motivation to run this risky experiment. So here you are reading this book.

To make one point very clear, by no means we expected a single book to cover really *all* aspects of stem cells; no single book can comprehensively describe a *revolution*. Yet, we wanted to cover those aspects of stem cells that we felt were more representative of this very broad field. We felt that this can be conceptually divided in two major categories: basic research and applications although, clearly, the two are deeply interconnected. This is the reason why *Stem Cells* is divided in two volumes. Volume 1 focuses on basic research, starting with an historical overview to then move to a series of chapters focussed on basic stem cell biology, tissue development during development and finishing with the main model organisms that are being used in our labs for stem cell research. Volume 2 continues with a more applicative twist, including stem cells in different tissues during adulthood, disease, therapy and their commercial use, with regulations and ethics connected to them. All chapters were contributed by internationally recognized experts in the respective fields. We are extremely grateful to all of them for sharing our enthusiasm and for contributing their time, knowledge and passion to bring together the many aspects discussed in this book. The merit of *Stem Cells* is all theirs; they are among the ones who fuel the *revolution*.

As our last note, our attention while editing *Stem Cells* was primarily addressed to students approaching this field and to more advanced investigators working on any topic related to stem cells. We truly hope that our work may contribute to the formation of those readers representing the

future generation of stem cell scientists. *Stem Cells* is dedicated to them; they are the ones who will carry on, and hopefully successfully conclude, this *revolution* for the benefit of society.



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# Gene Nomenclature

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The use of different symbols, names and aliases to refer to genes, mRNAs, and proteins has led over the years to an inconsistent literature in which different fields and authors tended towards the use of a specific nomenclature. To overcome this, official nomenclatures were adopted for any given model organism by the respective international committees including the Human Genome Organization (HUGO), the Mouse Genome Informatics (MGI), the Zebrafish Model Organism Database (ZFIN), and several others. In revising this book, we noticed however that strictly adhering to the official nomenclatures led to the paradoxical effect of inconsistency between chapters, and even inconsistency within sections of each chapter. For example, when referring to, say, the well studied insulin-like growth factor 1, the gene symbol assumed a number of different forms when referred to human, mouse, fish or chick (i.e.: *IGF1*, *Igf1*, *igf1*, or *IGF1*, respectively) (see Trends in Genetics, 1998, Vol. 14, Issue 11 for a comprehensive summary). Most notably, the universally accepted symbol of certain factors not always corresponds to its official nomenclature. In our book, this is certainly the case of the pluripotency factor Oct4 whose official name (*Pou5f1*) is ever barely used. Therefore, in trying to harmonize the use of gene and protein names throughout this book, we decided to use the official nomenclature adopted for mouse, primarily because mouse is typically the model organism more relevant for this book. Specific exceptions were made when human studies of clinical relevance were discussed, in which case human nomenclature was adopted (see below). Gene names and symbols were adopted according to the National Center for Biotechnology Information (NCBI) on its widely used and accessible PubMed database.

## Specific Example

Full gene name: insulin-like growth factor 1 (lowercase, not italicized for all species)

Gene symbol: *IGF1* (uppercase, italic for human); *Igf1* (initial letter uppercase, italic for other organisms)



Full protein name: insulin-like growth factor 1 (lowercase, not italicized for all species)

Protein symbol: Igf1 (initial letter uppercase, not italicized for all species)

mRNA/micro-RNA: are indicated with the same format of genes.

## **Links**

HUGO: <http://www.hugo-international.org/>

MGI: <http://www.informatics.jax.org/>

ZFIN: <http://www.zfin.org/>

PubMed: <http://www.ncbi.nlm.nih.gov/pubmed/>

# The History of Stem Cells

*Michel Morange*

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## SUMMARY

Stem cells are currently defined in terms of their capacities for self-renewal and generation of different types of cells, and great benefits are expected from their future use in regenerative medicine. The notion of a stem cell has evolved progressively since their first description in 1868. Initially, they were simply viewed as cells at a specific position in cell lineages. Their particular properties were described later by the study of hematopoiesis, and of teratocarcinomas. Whereas the use of blood stem cells preceded their characterization, embryonic stem cells were first considered as a tool to study early mammalian development, and their therapeutic utilization was only proposed when human embryonic stem cells were obtained in 1998. Other fields of research—plant development, regeneration, and cancer—contributed only marginally to the definition of stem cells. The present debate about the nature of stemness partially reflects the complex and tortuous history of the notion of stem cell.

## INTRODUCTION

Stem cells are able both to self-renew and to generate differentiated cells of one or multiple lineages. Asymmetric division is the preferred, but not only, way to give rise to two different progenies. Somatic stem cells are present in the adult and participate in the permanent renewal of cells of the blood, skin, and intestine as well as in the repair of tissues after injury such as the replacement of skin after burning. Embryonic stem cells (ES cells), derived

from the cells of the blastocyst, are multipotent and able to generate the different cell types present in a multicellular organism. Somatic, embryonic, and the more recently obtained induced pluripotent stem cells (iPS cells) generated by reprogramming of differentiated cells, are considered as the El Dorado of future regenerative medicine. Injection of cells differentiated *ex vivo* may provide a cure for the diseases resulting from tissue and cell degeneration. Stem cells also play a preeminent role in the morphogenesis of plants, and in the regenerative processes that have been described in some animal species for centuries. The recently described cancer stem cells might be the true actors in oncogenesis, and the cells that have to be specifically targeted in order to improve the hitherto incompletely effective therapies against this disease.

In this chapter, I will try to describe how this current and apparently coherent picture of stem cells progressively emerged. It is a long story spread over one and a half centuries, the result of different lines of research running in parallel but interacting little. The history of science is always more complex and tortuous than scientists imagine. This is particularly true in the case of stem cells. Casting light on this complexity is not pointless. It transforms today's apparently coherent picture into something more complex, the result of an unachieved process of the assimilation of competing visions. It explains some of today's debates as well as some of the experimental difficulties that are still encountered.

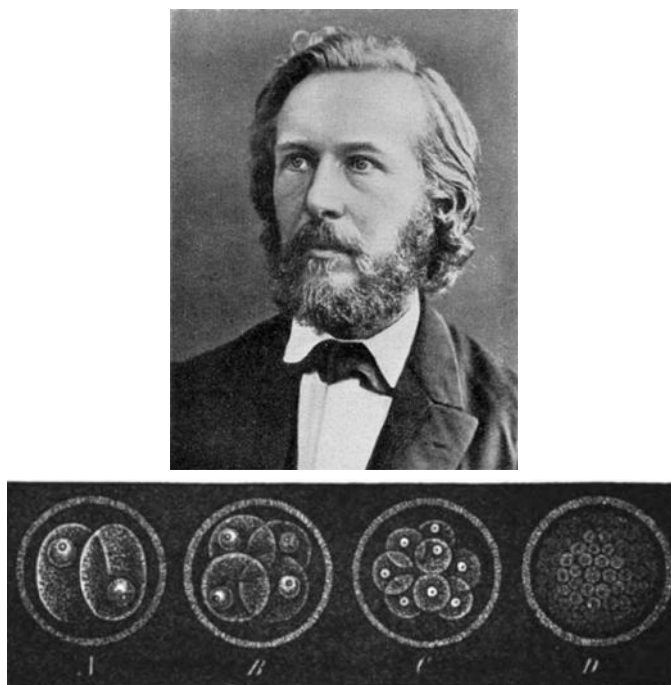
I have divided this historical presentation into four parts. The first deals with the emergence of the notion of stem cell, in relation with the studies on cell lineage that developed at the end of the XIX<sup>th</sup> century. The second is devoted to the emergence, or more precisely the acknowledgement, of the two fundamental characteristics of stem cells—self-renewal and multipotentiality—which simultaneously but independently became preeminent in the study of haematopoiesis and teratocarcinomas. The third part will concern the potential therapeutic use of stem cells associated with the two previous lines of research that emerged, as I will show, with a very different tempo. The last part will be devoted to three additional lines of research which joined the field of stem cell research quite late. They extended its validity, but despite early observations and efforts, did not participate in its construction: the study of regeneration, plant development, and the search for the stem cells of cancer. The delayed participation of these lines of research in the elaboration of the notion of stem cell requires an explanation, which is different in each case.

Parts of the history of stem cells have been already described by the scientists themselves or more recently by historians of science (Maienschein, 2003; Ramalho-Santos and Willenbring, 2007; Maehle, 2011). I have used information from these publications, which will be cited where appropriate. However, as far as I know, so far there has been no historical study that

tried to follow the rise and development of the concept of stem cell in the different fields in which it was used. Such a long-term (150 years) history reveals better than focused studies the interactions that existed (or not) between the different disciplines, as well as the complexity of what is presently placed under the banner of “stem cell”.

## STEM CELLS AND CELL LINEAGE

The term “stem cell” (“Stammzelle” in German) was introduced in a series of published lectures by Ernst Haeckel, Professor of Zoology at the University of Jena, and an active supporter of Darwin (Figure 1). He used the term in 1868 to designate unicellular organisms that he considered to be the evolutionary ancestors of multicellular organisms (Haeckel, 1868). In 1877, he again used the same term to designate the fertilized egg. This was only two years after Oscar Hertwig demonstrated that fertilization was the result of the fusion of the nucleus of the egg with that of the spermatozoon. The introduction of this new term was for Haeckel the way to emphasize the fact that the fertilized egg was very different from the maternal egg,



**Figure 1.** Picture of a 26 year old Ernst Haeckel taken on Christmass 1860 (top) and drawing of one of the first representations of an early embryo (bottom) from which the term *Stammzellen*, Stem Cells, was used (source: Wikimedia).

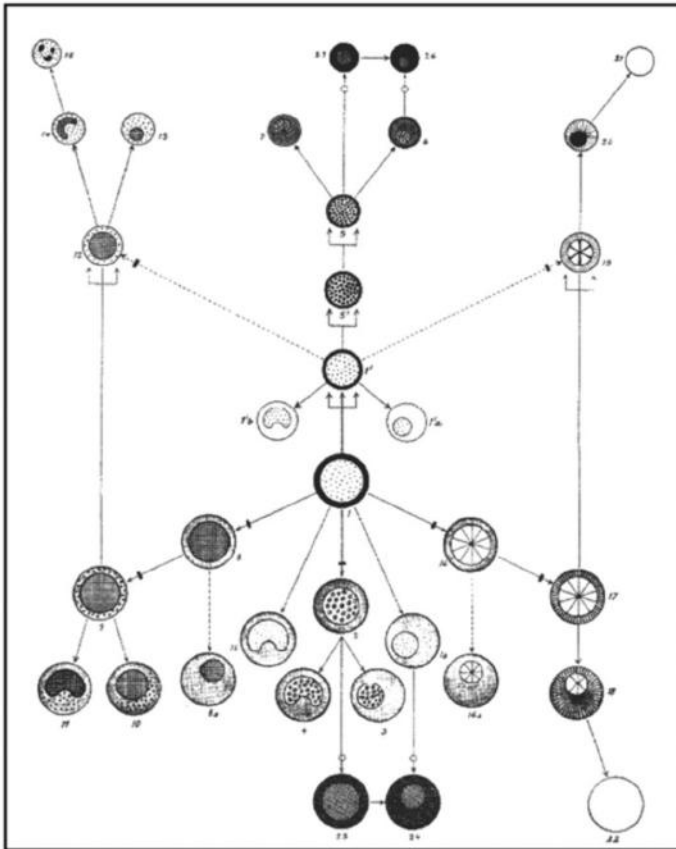
and that the newly formed cell was the precursor of all the cells of the future organism. The apparently two different uses of the term *Stammzelle* were identical for Haeckel. According to the biogenetic law that he had proposed, ontogeny recapitulates phylogeny: the *Stammzelle* is both similar to primitive unicellular organisms and the progenitor of the different cells that will form the adult organism.

The distinction made by August Weismann between the soma and the germen, and his emphasis on the continuity of the germ plasm through generations, led to a renewed interest in the term *Stammzelle*, but with a slightly different meaning. *Stammzellen* were presented in 1892 by Valentin Häcker, Weismann's assistant, as well as by Theodor Boveri in Munich, as the cells of the early embryo that were the source of the primordial germ cells. Theodor Boveri characterized these cells in his 1892 description of the early phase of the development of the model organism *Ascaris* (Boveri, 1892).

Both Häcker and Boveri focused their attention on the distribution of chromatin during these early cell divisions. Boveri was convinced that differentiation occurred in parallel with the loss of chromatin. This early work prompted, in the following years, a plethora of studies on cell lineages during early animal development, particularly in the United States, at the Marine Biological Laboratory of Woods Hole. Charles Whitman, Edmund Wilson and Edwin Conklin were the most famous researchers (Maienschein, 1978; Guralnick, 2002). They had all been, directly or indirectly, in contact with German embryologists. They were responsible for the English translation "stem cell" of the German term *Stammzelle*, and Wilson popularized it in his famous book *The Cell in Development and Inheritance* published in 1896 (Wilson, 1896).

This abundance of studies was the result of opposition between two different explanations of development. For the supporters of Haeckel, the biogenetic law was *the* explanation of early development, whereas most American biologists considered that development had to be explained by proximal mechanical causes operating during it. This cell lineage research programme faded in the 1910s because the rapidly accumulating data simultaneously showed a conservation of the first developmental steps, in agreement with the model of Haeckel, as well as the numerous variations that had occurred in different animal species. The latter could be explained by different mechanical forces acting on the early embryo. These studies confirmed the importance of asymmetric divisions, a mechanism initially described by Häcker, generating two cells with different fates (Figure 2A) (Häcker, 1892).

At the end of this period, the notion of stem cell had been firmly established, and some of its characteristics described. But stem cells were not considered as a population of cells, but as cells transiently forming during



**Figure 2.** One of the first representations of the hematopoietic lineage from Artur Pappenheim from 1896. One century later, blood stem cell lineages look essentially the same except for the use of color (source: Wikimedia).

development and as precursors of differentiated cells, intermediates in the long chains of cell lineages. Stem cells formed early in development and were the source of the progenitors of germ cells, a legacy of Weismann that we will encounter later in the description of the mechanisms accounting for the formation of teratocarcinomas.

Another meaning of the term stem cell was introduced in 1896 by Artur Pappenheim working at Virchow's Pathological Institute in Berlin on the formation of red blood cells. Using a comparative approach, he showed that there was a precursor cell common to the red and white blood cells that he named "stem" or "mother" cell (Figure 2) (Pappenheim, 1896, 1905). He described the existence of other types of stem cells in the different tissues of the organism. Both Wera Dantschakoff in Moscow and

Alexander Maximow in Saint-Petersburg proposed that the lymphocyte was the stem cell of blood cells. This unitarian view was supported by Ernst Neumann in Königsberg who had already proposed in 1868 that blood cells were produced in the bone marrow (Neumann, 1868). Paul Ehrlich, who had developed specific stains to distinguish the different cells present in the blood, proposed a dualist model, with two different stem cells, the lymphocyte and the leukocyte, and two different organs of production of these stem cells, the lymph nodes (and spleen) and the bone marrow. Among these different actors, Maximow is commonly considered today as the founder in 1909 of the haematopoietic stem cell concept (Maximow, 1909; Novik et al., 2009), although the term stem cell had been progressively introduced in the description of hematopoiesis before him. He obviously shared with the earlier introducers of the concept of stem cell that I described previously an interest in cell lineages. But the status of the hematopoietic stem cell was different, and Maximow made no reference to previous work. The question raised by Maximow and others was related to an important medical issue—the origin and nature of anaemias—far from questions on the mechanisms of early development. In both cases, however, stemness meant being part of an irreversible process. Boveri's model is emblematic of this conception: a stem cell is nothing more than a cell that has not yet lost a part of its chromatin.

## **SELF-RENEWAL AND MULTIPOTENTIALITY**

The simultaneous capacity of stem cells to self-renew and to generate different cell types was somehow implicitly included in the works that I have previously described on blood stem cells. But the first property of stem cells was not emphasized. What was considered most important was the multipotency, i.e., the capacity of the cells present in the bone marrow to generate the different cell types of the blood.

The dual nature of stem cells was to receive more attention three decades later, during the development of two highly different lines of research. The first concerned rare forms of tumors of the gonads called teratomas and teratocarcinomas, the first being benign and the second malignant. The distinction came later, and for a long while the term “teratoma” (sometimes “embryoma”) was used for both. The second line of research resulted as a prolongation of previous works on the characterization and isolation of the “true” haematopoietic stem cell.

## **From Teratocarcinomas to Embryonic Stem Cells**

Interest in teratomas and teratocarcinomas originated at the end of the XIX<sup>th</sup> century. In 1877, Julius Cohnheim, a former student of Virchow,

proposed that tumors originated from displaced embryonic cells that did not differentiate and remained present in the adult body (Cohnheim, 1877). This explanation of cancer contrasted with alternative models giving a preeminent role to infectious agents (bacteria, later viruses) or considering irritation of tissues as the main cause.

Cohnheim's ideas found strong support in the experimental work of Max Askanazy, Professor of General Pathology in Geneva (reviewed by Maehle, 2011). He was able to induce teratoma-like tumors by injecting embryonic cells into the abdominal cavity of rats. In 1907, he advocated that teratomas, rare tumors of the gonads, were the best demonstration of the link between development and cancer. He hypothesized that these tumors, which are a mixture of differentiated and undifferentiated cells, resulted from the misplacement during early embryogenesis of egg-like cells, which he called stem cells by reference to the work of Hacker and Boveri. This "blastomere theory" of teratomas was also supported by the pathologist Felix Marchand and the anatomist Robert Bonnet. Marchand also proposed an alternative hypothesis in which teratomas resulted from the accidental fertilization of polar bodies, the nuclear-like structures left aside in the egg during meiosis.

Although they were rare tumors, teratomas were actively studied in the following decades. The similarity between the events taking place during embryogenesis and what happened in the tumors was confirmed by the careful studies performed in Paris in the 1920s and 1930s by A. Peyron (Peyron, 1939).

One decisive step was made by Elizabeth Jackson and Austin Brues in 1941 by working on a teratoma from the C3H mouse strain (Jackson and Brues, 1941). Not only were they able to transplant the tumor during thirteen generations, but they demonstrated that the capacity to generate a tumor was due, and only due, to the small undifferentiated cells present in it—later called embryonal carcinoma (EC) cells. These experiments were complemented by tissue culture of the tumors that confirmed their pleiomorphic nature. Jackson and Brues hypothesized that this heterogeneous composition could be explained by an unequal division of the small EC cells, a notion identical to the asymmetric division described earlier.

A second decisive step was the discovery by Leroy Stevens in 1954 that teratomas occurred with a high frequency (more than 1%) in the testes of 129 strain mice (Stevens and Little, 1954; Andrews, 2002; Solter, 2006; Evans, 2011). The origin of these tumors was actively explored by Stevens and Barry Pierce (Stevens, 1962; Pierce and Beals, 1964). Their exclusive localization in the seminiferous tubules, as well as the possibility of generating tumors by ectopic transplantation of 12 to 15 day embryos, or simply of their genital ridges, suggested that they were the result of an anarchic development of



the progenitors of germ cells. When the embryos used for the graft bore a mutation preventing the formation of germ cells, no teratomas were formed (Stevens, 1967). But Stevens also showed that it was possible to induce the same type of tumors by transferring embryos of the late blastocyst stage (i.e., day 5.5 of development) to extra-uterine sites in syngeneic mice (Stevens, 1970). Reciprocally, injection of EC cells in the peritoneal cavity generated embryoid bodies, structures resembling early embryos. This resemblance between progenitors of the germ cells and early embryonic cells had been anticipated by Weismann.

From the mid-1960s, prolonging the work initially done by Pierce (Kleinsmith and Pierce, 1964), different cell lines were produced from these tumors by Boris Ephrussi (Finch and Ephrussi, 1967; Kahan and Ephrussi, 1970), Gordon Sato (Rosenthal et al., 1970), and later Martin Evans (Evans, 1972), François Jacob (Jacob, 1973) and many others (Solter, 2006; Evans, 2011). Some of these cell lines remained undifferentiated, and after injection they produced tumors in syngeneic mice containing only rapidly growing EC cells. Other cell lines were able to generate, after injection, both undifferentiated and differentiated cells. The most interesting of these cell lines were those which were able to differentiate *ex vivo*, spontaneously, as the PCC3 line studied in Jacob's lab (Nicolas et al., 1975), or after addition of a chemical inducer, e.g., the F9 cells that could be differentiated into cells resembling those present at the surface of the yolk sac by addition of cAMP and retinoic acid (Strickland et al., 1980).

It was not a question of chance that molecular biologists such as Ephrussi and Jacob turned to the study of teratocarcinomas at the end of the 1960s, and immediately produced cell lines. This was a time when many molecular biologists abandoned bacteria and bacteriophages—whose study has led to the concepts and models of molecular biology—for the study of multicellular organisms and their development. Before the elaboration of the tools of genetic engineering at the end of the 1970s, the direct molecular study of early mammalian embryos remained impossible. Teratocarcinomas, and the cell lines that had been isolated from them, offered the amount of material required for a molecular study of early development (Jacob, 1978).

Reciprocally, the molecular studies rapidly confirmed the similarities between cells of the early embryos and EC cells. A protein, present at the cell surface of the early embryo at the morula stage, was also expressed by the F9 EC cell line (Jacob, 1977). This so-called F9 antigen was shown to play a decisive role in the compaction of the embryo that precedes the formation of the blastocyst and the first differentiation events. An additional assumption that the F9 antigen was coded by a gene complex called the T-locus was not further confirmed (Morange, 2000). Nevertheless, the early results from the laboratory of Jacob, and from other laboratories, strengthened

the correlation between the events occurring in embryogenesis and the phenomena observed in teratocarcinomas, and during the differentiation of EC cells.

Rapid progress in the 1960s and 1970s of methods for the manipulation of early embryos *ex vivo* (mostly mouse) offered additional evidence for the similarity between teratocarcinomas and embryos. At the end of the 1960s, three different techniques were described for the production of chimeric embryos by the association of embryonic cells of different origins. These techniques were used to explore the relation between EC cells and cells from early embryos. In 1974 and 1975 different groups introduced EC cells into blastocysts (Brinster, 1974; Papaioannou et al., 1975; Mintz and Illmensee, 1975). After implantation of the chimeric embryos into the uterus of surrogate mothers, mosaic animals were obtained with different tissues derived from EC cells.

These experiments were an additional confirmation that teratocarcinomas were an experimental system well adapted to the study of development (Jacob, 1978; Martin, 1980). They also confirmed the existence of an antagonistic relation between oncogenesis and differentiation: the EC cells injected into the blastocysts differentiated and lost their capacity to generate tumors. Among the numerous models proposed in the 1960s and 1970s to explain the formation of tumors, one of the most favoured was that cancer resulted from a dysregulation in gene expression leading to a reactivation of genes normally expressed during development, and generating a rapid rate of cell division (Morange, 1997). Therefore, teratocarcinomas were also an experimental system appropriate to outline the fundamental characteristics of cancer (Pierce, 1967; Martin, 1975). They were also a valuable experimental system in the search for drugs able to trigger differentiation of cancer cells, and concomitantly to promote a loss of their tumorigenic power (Braun, 1965; Pierce, 1967).

This accumulation of data showing the similarity between EC cells and the early cells of the embryo, together with the active participation of EC cells microinjected into blastocysts in the normal development of mouse embryos, was the trigger that led Martin Evans, Matt Kaufman, and Gail Martin to derive in 1981 embryonic stem (ES) cells from cultured blastocysts (Evans and Kaufman, 1981; Martin, 1981).

These two experiments were far from being the first attempts to derive embryonic cell lines from blastocysts. In 1975, Michael Sherman gave a comprehensive description of these efforts and of their partial success (Sherman, 1975). These efforts had been made by the same researchers who tried to establish the conditions for the *ex vivo* culture of embryos. This led Robert Edwards, the author of the first *in vitro* fertilization study in humans and the “father” of the first “test-tube baby”, to consider that these efforts underpinned the production of the first ES cells in 1981

(Edwards, 2001). It is obvious that the knowledge that had been acquired on the manipulation of early embryos, and the culture media that had been selected, were necessary for the success of the 1981 studies. But what emerges also from the description by Edwards of these early experiments is a confusion between two distinct objectives: the reproduction *in vitro* of normal embryonic development as opposed to the production of stable immortal cell lines with properties similar to EC cells (Edwards, 2001; Brandt, 2012). The merit of the researchers who had been working on EC cells was to have clearly distinguished the two characteristics of stem cells, their capacity to self-renew in an undifferentiated state and to generate different types of differentiated cells.

The term 'stem cell' was used very late by researchers working on teratocarcinoma, at the time when embryonic stem cells were produced. One likely reason is that this term was already used in studies on hematopoiesis (see below) in which such physiological, well-regulated process did not seem to fit with the anarchic development of teratocarcinomas (Brandt, 2012).

## **From Bone Marrow to Haematopoietic Stem Cells**

There is a general agreement in the literature that the experiment performed by James Till and Ernest McCulloch in 1961 led to the characterization and isolation of haematopoietic stem cells (Till and McCulloch, 1961).

In this experiment, the two researchers injected cells isolated from bone marrow into syngeneic mice that had previously received a supralethal dose of irradiation. They observed the formation of well visible nodules in the spleen containing both erythroblasts and myeloblasts. They hypothesized that each of these nodules was founded by one unique precursor cell, a colony-forming unit. The hypothesis was confirmed in a further publication (Becker et al., 1963), using a protocol that had been previously devised to distinguish, after transplantation, between the cells of the donor and those of the recipient (Ford et al., 1956; see below). It appeared that only a very small fraction of the bone marrow cells that had been injected (about 1/1000) was able to generate such colonies.

Some historical studies (e.g.: Fagan, 2007) mentioned that early evidence for the possibility of transferring blood stem cells came from the observation that bovine twins of a different genotype could nevertheless have identical blood cells. The hypothesis was that the blood stem cells of one of the twins had been replaced by the stem cells of the other through the vascular anastomoses that exist between twins (Owen, 1945). As we have seen, the concept of blood stem cells was first enunciated much earlier (Pappenheim, 1896, 1905) and, as recognized by its authors (McCulloch and Till, 2005), the result of the 1961 experiment was serendipitous. The

objective of the experiments reported in the article was different: it was to measure the sensitivity to radiation of blood stem cells and to compare it with the sensitivity to radiation of other cells. McCulloch and Till's article belonged to a long series of publications comparing the effect of X-rays on mammalian cells (one of the first being Puck and Marcus, 1956).

The meaning of such experiments can only be understood in the particular context of the 1940s and 1950s. The sensitivity of organisms and tissues to radiations was a major concern after the atomic bombs of Hiroshima and Nagasaki (Beatty, 1991) and following some accidents that had occurred during the manipulation of radioactive materials. The anemias resulting from the treatment of cancer by irradiation were also a major medical concern. Efforts were made very early to find some way to prevent or repair the damage due to irradiation (Kraft, 2009). Some chemical agents were tried, without conclusive effects. In 1949, Leon Jacobson made an observation that had a huge impact (Jacobson et al., 1949): protection of the spleen during irradiation had an important beneficial effect. He later confirmed this major effect by transplanting the spleen from a syngeneic donor animal to an irradiated recipient and showing the benefits for the latter. Two different interpretations were given of these experiments: some considered that the spleen produced a humoral agent (maybe a hormone) whereas others favoured a cellular factor.

In parallel, but independently, Egon Lorenz showed that transplantation of bone marrow also facilitated recovery from radiation injury (Lorenz et al., 1951). The conclusion was that the recovery factor was present not only in the spleen but also in greater amount in the bone marrow. Lorenz and his colleagues favoured the hypothesis that it was a cellular factor. The proof was finally provided in 1956 by John Loutit and his colleagues by using a chromosome marker to distinguish the cells of the donor from the cells of the recipient. They demonstrated that the recovery paralleled the presence of donor cells in the recipient animal (Ford et al., 1956).

Meanwhile, an obstacle to the cellular nature of the recovery factor had been discarded: the rule, established by Alexis Carrel in the 1930s, that there was an absolute transplantation barrier between organisms. Peter Medawar demonstrated that tolerance to foreign cells could be actively acquired (Billingham et al., 1953) and, in 1955, it was shown that transplantation of bone marrow to irradiated animals facilitated the subsequent graft of skin from the same donor (Main and Prehn, 1955). The previous observations suggested that the bone marrow stem cells might be more sensitive to irradiation than other cells. The results of the 1961 experiment did not confirm this hypothesis. While this experiment was the end of a long series, it was also the beginning of a new story. Till and McCulloch very rapidly realized that the experimental protocol that they had devised was a simple and efficient way to estimate the proportion of stem cells present in a crude

cell population. It provided a quantitative measure of the purification factor of stem cells from the crude cell preparations.

In subsequent publications, they were able to show that each nodule contains cells able to generate new nodules, which led them to clearly distinguish the two characteristics of stem cells: self-renewal and multipotency revealed by the heterogeneity of cells present in each nodule (Siminovitch et al., 1963). Interestingly, they proposed a stochastic model to explain the heterogeneity of the nodules (Till et al., 1964).

While the experiment of 1961 indicated the road to the purification of blood stem cells, two scientific and technological developments had to be accomplished for this purification to become feasible. The first was the development of fluorescence-activated cell sorting technology, which allows the separation of cells bearing different fluorescent labels. The second was the characterization of the molecular markers linked to the differentiation of hematopoietic cells. These molecular markers of differentiation were progressively described in the 1970s and 1980s. This explains why Irving Weissman's group, which was engaged in the molecular characterization of the different steps in the differentiation of hematopoietic cells, was the first to obtain "pure" stem cells in 1986 (Muller-Sieburg et al., 1986; Spangrude et al., 1988; Fagan, 2007 and 2010).

The debate was not over though. It has been argued that Weissman's publication had been predated by another study (Visser et al., 1984). Moreover, by using retrovirus-mediated gene transfer to label hematopoietic stem cells, it was shown that the population of so-called "pure" hematopoietic stem cells was actually heterogeneous (Lemischka et al., 1986), a result which was later accepted by Weissman's group (Morrison and Weissman, 1994). Nevertheless, Weissman still maintained that the heterogeneity originated in the different hierarchical positions of the cells during the differentiation process, and not from stochastic events as previously suggested (Till et al. 1964; Lemischka et al., 1986).

Although both studies on hematopoiesis and teratocarcinomas permitted the identification of the main characteristics of stem cells, self-renewal and the capacity to generate different cell types, the work on teratocarcinomas added something more: the generation of immortal cells, a prerequisite for a therapeutic use of stem cells. The progressive emergence, since the beginning of the XX<sup>th</sup> century, of the notion of cellular immortality was the consequence of the efforts made to cultivate cells and tissues *ex vivo*. Immortality was an issue that remained high on the agendas of biologists throughout the XX<sup>th</sup> century (Landecker, 2007) and still has a strong impact on the vision of stem cells by the lay public.

## THE THERAPEUTIC USE OF STEM CELLS

Today's definition of stem cells incorporates their potential use in regenerative medicine. In the previous section, I described the two independent pathways which led in the 1980s to the isolation of the first pure populations of embryonic and hematopoietic stem cells. Strangely enough, whereas the transplantation of bone marrow preceded the isolation of hematopoietic stem cells, seventeen years separated the isolation of mouse embryonic stem cells from that of human embryonic stem cells and from a clear perception of the therapeutic potential of these cells.

The first transplantation in humans of bone marrow cells occurred in 1939, without success (Osgood et al., 1939). As we have previously mentioned, Ford and colleagues had shown in 1956 that donor cells from spleen or bone marrow could survive in a recipient irradiated mouse (Ford et al., 1956). However, the spectacular bone marrow treatment used in France by Georges Mathé on accidentally irradiated nuclear workers brought them little, if any, benefit (Mathé et al., 1959). Twenty years were necessary, and many obstacles had to be overcome, before bone marrow transplantation became feasible (Thomas et al., 1957; Thomas, 1990). These efforts were particularly justified by the absence of any efficient treatment of leukemias, a disease touching young people. The impetus towards this heroic form of medicine was also given by the first success obtained in kidney transplantation between twins (reviewed by Kraft, 2009).

The first obstacle was the rejection of foreign cells by the recipient, which explains why success was achieved in syngeneic animals, between twins in humans, and only partially and transiently in irradiated organisms in which the cells of the immune system had been destroyed. Progress was made possible by the description by Benacerraf, Dausset, and Snell (Nobel Laureates, 1980) of the human histocompatibility complex. The second obstacle was due to the inverse reaction, the graft versus host reaction, which found a partial solution in the elimination of T-lymphocytes from the injected cells, but mostly from the progressive discovery of effective immunosuppressive drugs. It was not until the 1990s that a truly effective drug, cyclosporin, was released. This solution generated a third obstacle, which was painfully overcome: transplanted patients, submitted to immunosuppressive treatments, were highly sensitive to opportunistic infectious agents. A long series of efforts limited possible sources of contamination and helped find the right treatments for these infections. It was only in the 1990s that the injection of bone marrow cells became an effective therapy for patients who had been submitted to high doses of irradiation for medical reasons or by accident, for patients suffering from leukemias and from some rare blood diseases.

This long story is totally independent of the progressive characterization and isolation of hematopoietic stem cells that I described previously. The transplantation of bone marrow cells was justified by the existence, within the population, of stem cells; but the precise nature and abundance of these cells was not a major issue.

Interestingly, it was only in the 1990s that the isolation of bone marrow stem cells became a major issue for new applications of bone marrow transplantation. The objective was to cure genetic diseases such as thalassaemias by reintroducing fully functional copies of the genes in the patients in which they were not functional. The success of this manipulation required the new functional genetic information to be introduced into the stem cells, which was the only way to stably correct the genetic deficit. Gene therapy was much more difficult to develop than had been anticipated and its therapeutic value has not yet been fully confirmed.

Concerning the therapeutic use of embryonic stem cells, it is striking that not one reference was made to a possible therapeutic use, and to the production of human ES cells, in either of the two articles in which the production of the new cells was described (Evans and Kaufman, 1981; Martin, 1981) or in the comments that accompanied them (Hogan, 1981). These cells were seen as a wonderful tool to extend the work that had previously been done with EC cells in order to understand the mechanisms of differentiation and development. These cells were also seen as useful for exploring the antagonistic relation between differentiation and development, and cancer. The title of the comments written by Brigid Hogan on the article of Evans and Kaufman emphasized that ES cells were only a better tool for extending the studies that had been performed before “from embryo to teratocarcinoma in tissue culture” (Hogan, 1981). One of the criteria used to characterize these cells was their capacity to generate tumors when injected into a syngeneic animal. They were considered as more reliable than the EC cells that had remained in culture for long periods and had probably lost some of their original characteristics.

But the major interest for these new cell lines was the possibility to extend genetic studies of development. These cells were expected to be able to form chimeric embryos and to participate in their development as well as, and even better than, EC cells. It was also anticipated that they would be much more efficient than EC cells in the formation of germ cells. In this way it would be possible to introduce new genetic information and to test the effects on the development and characteristics of the organisms resulting from these manipulations.

The results exceeded the hopes when, at the end of the 1980s, Mario Capecchi (Nobel Laureate, 2007 together with Martin Evans and Oliver Smithies) devised a technique using homologous recombination to replace a gene by an altered copy of it (Capecchi, 1989). By using this technique, it

became possible to test *in vivo* the role of the “developmental genes” that had been characterized from 1984 onwards, in most cases by their homology to genes in *Drosophila* but without any precise idea of their function. The simultaneous use of ES cells and homologous recombination generated a wealth of knock-out and knock-in animals, which still today remains one of the major tools used by biologists to determine the function of genes in mammals. Mutations responsible for human genetic diseases have been introduced into the murine genome, giving rise to animal models of these diseases. Their study, including that of models of neurodegenerative diseases, Alzheimer’s and Parkinson’s, has utterly transformed the knowledge of these diseases and has permitted rapid testing of newly designed drugs.

The importance of ES cells for a genetic description of mammalian development probably explains why other potential uses of these ES cells were not immediately considered. The oncogenic potential of these cells was also obvious to the researchers familiar with the teratocarcinoma system and raised a serious question mark over their potential use in therapy.

Another reason is that degenerative diseases—those presently targeted by the use of ES cells—did not receive in these years the same attention as today when aging, together with its associated diseases, is considered as a major challenge for our societies. But the lack of interest in the therapeutic use of ES cells originated mainly from the fact that other therapeutic approaches were preferred in these years including organ transplantation and gene therapy (Morange, 2006).

The production of human ES cells required previous assays on rhesus monkeys (Thomson et al., 1995) and modifications of the protocols which had been used in 1981 (Thomson et al., 1998). Still, these minor technical adjustments do not explain the long time interval separating these studies. In 1998, embryonic germ cells were also produced by culturing the precursors of germ cells (Shamblott et al., 1998). They had properties very similar to those of stem cells: a confirmation of the results previously obtained by the study of teratocarcinomas.

The efforts that were deployed in the mid-1990s to produce human ES cells were the result of the difficulties encountered in organ transplantation and of disappointing results of early gene therapy. The problems associated with organ transplantation were not technical since most of them have been solved satisfactorily. The main concern was the rarity of donors. The extensive efforts that have been made to increase the number of donors by appealing to relatives, extending the range of deaths compatible with the gift of an organ, and the optimized use of organs, highlight the limits whose violation may challenge the ethical values of our societies (see Part X). The expressions which were initially used to designate this new therapeutic



approach using ES cells, “transplantation therapy” (Gearhart, 1998) and “transplantation medicine”, explicitly referred to organ transplantation.

Gene therapies have encountered many difficulties that were not anticipated when the first experiments were initiated. Probably also a genetic solution no longer appeared appropriate for diseases that are not genetic. Cell therapy and regenerative medicine are presently more compatible with the present conceptions of cell biology than with the previously over-optimistic visions about molecular genetics. It was not by chance that the study that led to the production of human ES cells was done in part by a biotechnology company (Geron) that had been heavily involved in gene therapy but had decided to abandon it. The same year that human ES cells were produced, researchers from Geron reported that the introduction of the telomerase gene into recipient cells could prevent their senescence (Bodnar et al., 1998). Combating tissue degeneration by rejuvenating cells *in situ* through genetic engineering was the strategy pursued by Geron but later abandoned in favour for the introduction of ES cell-derived cells into degenerated tissues. Interestingly, telomerase, that was used to rejuvenate cells, was also used as a tool to check the immortal character of the newly produced human ES cells. The contrast between the articles in 1981 and 1998 is striking since the therapeutic use of ES cells had become the major argument used to emphasize the scientific accomplishment and the main message transmitted by its commentators (Gearhart, 1998; Marshall, 1998).

Two other scientific developments occurred simultaneously with the production of ES cells and contributing to its impact. The first was the birth of Dolly in 1997, the first experimental mammalian clone (Wilmut et al., 1997). When putting together the two technologies, it became theoretically possible to generate ES cells by nuclear transfer that are genetically identical to, and therefore fully compatible with, the patient who will receive the cells after transplantation. It also became possible to generate genetically modified ES cells that should be able to repair organs of individuals suffering from genetic disease. The second discovery was the evidence for the existence of adult stem cells even within those tissues that were historically considered to lack any capacity of renewal, most notably the adult brain (Altman and Das, 1965). Although embryonic and adult stem cells have often been contrasted, because the ethical issues raised by their manipulation are very different, the existence of adult somatic stem cells provided strong support for regenerative medicine by showing that therapeutic regeneration may be considered as an extension of a “natural” process, i.e., the manipulation of processes that is already present within organisms.

## THE BELATED SUPPORT FOR THE STEM CELL CONCEPT

The concept of stem cell is commonly used today to explain the regenerative power of plants and of some animals, as well as the characteristics of tumors and the possible new therapeutic approaches to cancer. Paradoxically, these fields of endeavour were not active players in the emergence of the concept of stem cells although they have contributed to their present importance. The reasons for this are worth considering.

### Meristems: Cell Stems in Plants

The term “meristem” was introduced in 1858 by the botanist Carl Wilhelm von Nägeli to designate a plant tissue “from which the entire organ is originally formed, and which will also remain active later, sometimes for the entire life” (Nägeli, 1858; quoted in Scofield and Murray, 2006). Carl Nägeli is also infamous in the history of biology for not having paid enough attention to the articles that Gregor Mendel sent him and in which the laws of genetics were described for the first time. Unlimited *ex vivo* growth of cells derived from root meristems was obtained in the 1930s (White, 1934). The description of cells present in meristems exactly fits the definition given for stem cells.

However, the origin of the word “meristem” is different from that of the word “stem” (Ramalho-Santos and Willenbring, 2007). And the existence of “plant stem cells” has not helped the emergence of the concept of stem cells. It was only in 1992 (Francis, 1992), and later in 1997 (Hudson and Goodrich, 1997), that the expression “stem cell” was explicitly used by botanists to describe the cells present in meristems.

I propose three reasons for this lack of interest. The first is the difficulty in exchanging models between plant and animal biology, which has, with some exceptions, been a constant in the history of the life sciences. The second is that the notion of cells able to regenerate the whole plant is obvious for botanists, originating as it does both in centuries-old agricultural practice and in the more recent experiments of cell biologists. As Scofield and Murray nicely put it: “the pluripotent nature of cells in many plant tissues under the right culture conditions suggested that it was a less obviously useful term in defining a particular attribute of special types of cell” (Scofield and Murray, 2006). But, in addition, the “true” nature of stem cells in plants remains unclear: are they “stem cells” or rather cells able to dedifferentiate to regenerate the different tissues of the whole plant?

## **The Study of Regeneration**

A similar difficulty, and its long history, also explains why the study of regeneration has not been more involved in the emergence of the notion of “stem cell”. Regeneration fascinated the naturalists of the XVIII<sup>th</sup> century after the discovery of hydras and the description of their fantastic power of regeneration by Abraham Trembley in Geneva (reviewed by Cooper, 2003). It was an important subject in the debates opposing preformationists and epigeneticists at that time. It was also, through Blumenbach, an impetus for Immanuel Kant to clearly distinguish the properties of organisms from those of inanimate objects.

One century later, regeneration was a challenge for those who tried to elaborate a theory of heredity. Darwin included it in the model of pangenesis he elaborated in 1868. Thomas Morgan spent many years studying regeneration before his conversion to genetics. He saw the study of regeneration as an alternative and original way to address the question of development. Despite numerous experiments, he was unable to reach firm conclusions (Morgan, 1901). He hypothesized the existence of pluripotent stem cells to explain regeneration and returned only briefly to this question in his later works.

It is only recently that the question of regeneration has been subject to renewed interest. Maybe some regenerative capacities are present in our tissues as a remnant of the regenerative powers of simpler forms of life that are waiting to be unmasked. The simplest explanation of the phenomenon of regeneration would be in the existence of stem cells, silent in normal conditions and activated when a regenerative process is required. Recent studies often do not reveal such a simple scenario. The mechanisms of regeneration appear diverse and dedifferentiation of differentiated cells appears to have a role as important as that of stem cells (Sanchez-Alvarado and Tsonis, 2006).

## **Evidence for the Existence of Cancer Stem Cells**

The last field of research where the place and importance of stem cells is actively discussed today is cancer (Kraft, 2011). Tumors might originate from a small distinct subpopulation of cells. These cancer stem cells might be the result of mutations in the stem cells present in the organism. The notion of cancer stem cells originated in two publications by John Dick in 1994 and 1997 in which he describes the transfer of human acute myeloid leukaemia to recipient immunodeficient mice by a well-characterized subpopulation of leukaemic cells expressing specific markers (Lapidot et al., 1994; Bonnet and Dick, 1997) and four years later these first observations were generalized (Reya et al., 2001). Cancer stem cells were shown to use the same signalling

pathways and to express the same molecular markers as normal stem cells. In 2003, similar observations were done on breast cancer, an example of a solid tumor (Al-Hajj et al., 2003; Dick, 2003). Since these early observations, the existence of cancer stem cells has been described in many different types of tumors. The protocol used for the isolation (and definition) of cancer stem cells was very similar to that used by Till and McCulloch in 1961. What was different, and the result of the progress made in molecular cell biology, was that the cancer stem cells were defined not only by their capacity to generate a tumor, but also by their molecular signatures defined as the markers specifically expressed at their surface.

The evidence that, within tumors, only a small fraction of cells are oncogenic was not new. More than fifty years before EC cells had been shown to be the stem cells of teratocarcinomas (discussed above). But it is also true that teratocarcinomas had characteristics distinguishing them from most tumors: the heterogeneity of cells found within these tumors contrasts with the apparent homogeneity of most cancers. The idea that cancer stem cells were derived from stem cells was also not new, and I have already mentioned that it was first introduced by Cohnheim at the end of the XIX<sup>th</sup> century even if this hypothesis was criticized in the 1920s (Maehle, 2011). What probably explains the renewed interest in these ideas is the increasingly evident limits of the traditional approaches to cancer targeting the rapidly dividing cells of the tumor. The new hypothesis designates a subpopulation of tumor cells to be targeted and therefore brings with it the hope of more effective treatments.

## **CONCLUSIONS AND FUTURE PERSPECTIVES**

Since the term “stem cell” was introduced in 1868, different properties and characteristics have been attributed to these cells. The current definition of stem cells emphasizes their pluripotency and self-renewal capacity, as well as their potential use to regenerate tissues. This definition has been progressively constructed during the complex history that I have described above.

This process of construction is probably not yet finished. Two different ways to define stem cells are alternatively (or simultaneously) used: the molecular signatures that characterize them and their capacity to generate different cell types. Both definitions can converge, as in the case of the initial characterization of cancer stem cells but this dual definition is not always used or immediately considered as obligatory. One can imagine, for instance, that the capacity to generate certain types of differentiated cells is not the property of a single cellular state, with well-defined molecular signatures, but of a population of cell states between which there is rapid interconversion, each state having its specific molecular signature.

The correlation between the position of a cell in a cell lineage and its cell stem “power”, i.e., its capacity to generate a group of differentiated cell types, had only been marginally challenged since the term stem cell was first introduced at the end of the XIX<sup>th</sup> century to designate a cell appearing at the highest position in the first established cell lineages. This correlation was completely revolutionized by the production of induced pluripotent stem cells in 2006 (Takahashi Yamanaka, 2006). The consequences of this dramatic change have probably not been fully appreciated yet.

Finally, in most cases so far considered, what was important for the stemness of a cell was its “history”, natural by lineage or artificial by genetic manipulation in the case of induced pluripotent stem cells. The environment of the stem cells, what was called “their niches” (reviewed by Spradling et al., 2001), was considered as important only because it could favour or prevent the maintenance of stemness. It is possible to imagine a different model in which it is the environment that is responsible for the formation of stem cells. The production of stem cells, and their characteristics, would be causally due to the niche in which they are located. Such a causal role of the environment seems obvious in the case where stem cells are generated by dedifferentiation of previously differentiated cells (as perhaps in some cases of regeneration). With the production of induced pluripotent stem cells, ascribing a major role to the environment would clearly lead to a new concept of stem cells (Lathia et al., 2011) and stemness.

These issues are reminiscent of the debate that occurred at the end of the XIX<sup>th</sup> century and at the beginning of the XX<sup>th</sup> century between Wilhelm Roux and Hans Driesch on the regulatory capacities of early embryos. The place and limits of plasticity in the development and functioning of organisms has always been at the core of discussions between biologists. The still debated importance of the dedifferentiation and trans-differentiation processes in the formation of stem cells is a part of this general debate.

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**PART I**

**BASIC STEM CELL BIOLOGY**

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# Cell Cycle Regulation of Pluripotent Stem Cells

*Amar M. Singh and Stephen Dalton\**

## SUMMARY

Pluripotent stem cells are defined by two criteria, the ability to undergo self-renewal and multi-lineage differentiation. The maintenance of stem cell self-renewal is based on the rapid progression through the cell cycle, placing cell cycle regulation as a central component of stem cell maintenance. Interestingly, pluripotent stem cells have many unique cell cycle characteristics that distinguish them from somatic cells. Most notable of these is the unique structure of their cell cycle profile. Unlike somatic stem and differentiated cells, pluripotent stem cells possess a short G1 phase and have an unusually large percentage of cells in S phase. The regulatory mechanisms that promote this unique cell cycle profile are described, along with the potential purpose of having this cell cycle structure and what it may mean for maintaining pluripotency and self-renewal. Importantly, the mechanisms that regulate the cell cycle of mouse pluripotent stem cells is broadly different from somatic or differentiated cells, and also appear

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*List of abbreviations after the text.*

to be quite different from human pluripotent stem cells. Many of these mechanisms are only now being understood but involve a complicated scheme of regulation at multiple levels, including transcriptional and post-transcriptional regulation, such as protein modifications and miRNA-based translational repression. In the past, overcoming technical challenges in evaluating cell cycle mechanisms have proven difficult. However, numerous new tools have been developed that will aid researchers to fully dissect the regulatory events necessary to promote the cell cycle of pluripotent stem cells.

## **INTRODUCTION**

The mechanisms that regulate the cell cycle, proliferation, and cell division of eukaryotic cells have captivated biologists for decades. Research in this area is of widespread interest and importance for understanding embryonic development, tissue homeostasis, and various pathologies, including cancer. While much of the early work in cell cycle regulation was performed in yeast, it is now clear that many of these discoveries can be translated to all vertebrate and mammalian organisms. The culmination of these discoveries resulted in the awarding of the Nobel Prize in physiology or medicine to Drs. Leland Hartwell, Tim Hunt, and Sir Paul Nurse in 2001. In this chapter, we will begin with a basic introduction of the cell cycle and pluripotent stem cells and continue by describing the unusual properties of the cell cycle of pluripotent stem cells. We will further discuss recent discoveries and control mechanisms of the cell cycle in pluripotent cells, such as regulation by Myc and miRNAs, and conclude by addressing some of the major questions that are still unresolved.

## **GENERAL FEATURES OF THE CELL CYCLE**

Since numerous reviews and textbooks have been written about the eukaryotic cell cycle, we will only provide a general framework here. For a more in depth review we recommend several recent reviews (Bartek and Lukas, 2001; Dimova and Dyson, 2005; Malumbres and Barbacid, 2009; Weinberg, 1995).

The mitotic cell cycle is divided up into 4 phases, two Gap phases (G1 and G2) with an intervening DNA Synthesis phase (S phase) and concluding with Mitosis (M phase). Together G1, G2, and S are known as interphase and take up the majority of the time of the cell cycle, which generally ranges from 8–15 hours for mammalian cells, depending on the cell type. M phase is relative short for most cell types (~ 1 hour) and culminates with cytokinesis and the generation of two daughter cells. Following cell division, cells may

exit the cell cycle from G1 and become quiescent (designated as G0), the best-characterized example is that following mitogen deprivation. Re-entry back into the cell cycle following growth factor withdrawal is dependent on the addition of fresh mitogens that function by activating the cell cycle machinery. Progression through the cell cycle is controlled by various genomic checkpoints such as at the G1/S and the G2/M transitions (Bartek and Lukas, 2001). Should the cells activate one of the checkpoints, the cell cycle will arrest and will either repair DNA or undergo apoptosis. Another important hallmark of the cell cycle is the restriction point (R-point) in late G1 (Planas-Silva and Weinberg, 1997). Once past the R-point or “point of no return”, a cell is fully committed to enter S phase and begins duplication of its genome. If the appropriate growth stimuli are not present, cells will not proceed past the R-point and enter S phase, but instead will enter G0.

Control of the cell cycle is regulated by a family of serine/threonine protein kinases known as cyclin-dependent kinases (CDKs) (Malumbres and Barbacid, 2009). CDKs are composed of a CDK catalytic subunit and a regulatory subunit known as a cyclin. Activity of the different CDK-cyclin complexes oscillate as cells progress through the cell cycle, in part because the expression of cyclins changes throughout the cell cycle, hence modulating the activity of the CDK catalytic subunit. As a cell progresses through each phase of the cell cycle, a particular CDK-cyclin will control the entry into the next phase. In fact, each phase can be defined by which specific CDK-cyclin complex is active at that particular time (Malumbres and Barbacid, 2009). For example, CDK4/6-cyclin D regulates the progression from early to late G1, while CDK2-cyclin E regulates the transition from G1 to S. In general, phase-specific CDK complexes function by phosphorylating substrates required for the different cell cycle transitions. For example, the mitotic CDK1-cyclin B complex phosphorylates substrates required for M phase progression and restriction of cytokinesis while the S phase complex CDK2-cyclin E phosphorylates substrates required for DNA replication. In addition to CDK-cyclin complexes, the CDK inhibitor (CDKI) proteins can block progression through each phase of the cell cycle (Sherr and Roberts, 1999). Examples of CDKIs include members of the CIP/KIP and INK4 family that inhibit CDK-cyclin complexes. For example, P21<sup>CIP1/KIP</sup> (CDKN1A) can directly bind and inhibit cyclin A/E complexes, thereby blocking the progression from G1 to S, while INK4 family members, such as P16<sup>INK4a</sup> (CDKN2A), have functions associated with inhibition of CDK4/6-cyclin D complexes (Sherr and Roberts, 1999).

## **E2F, RB1 and Control of the G1/S Transition**

The E2F family of transcription factors plays a critical role in the regulation of the cell cycle (G1/S transition) and DNA synthesis (Dimova and Dyson,

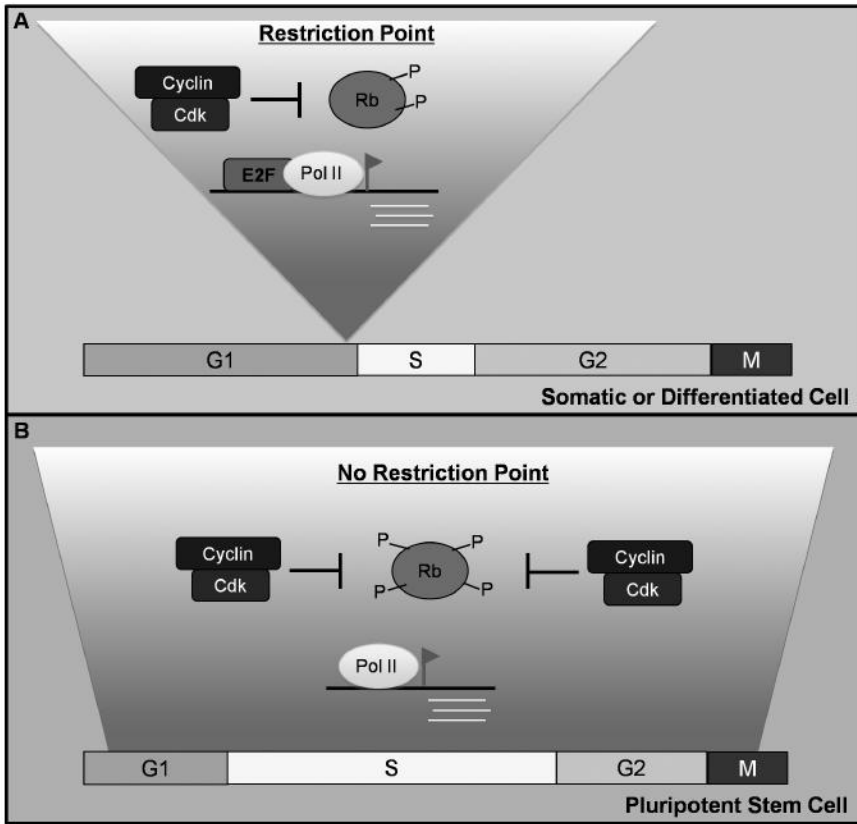
2005). In fact, a majority of the proteins involved in the initiation and elongation of DNA synthesis and G1/S progression, such as proliferating cell nuclear antigen (PCNA), DNA polymerase  $\alpha$ , and cyclin E are under the transcriptional regulation of E2F. Therefore, it is not surprising that deregulation of E2F-dependent transcription may arise in deregulation of cell growth and tumor formation. This is particularly evident in cases of cervical cancer caused by the human papillomavirus, whose E7 protein perpetuates enhanced E2F activity (Morris et al., 1993).

At the heart of E2F regulation are the pocket proteins retinoblastoma, RB1, RB2, and RB3 (Harbour and Dean, 2000). RB family of pocket proteins have essential roles in controlling the G1/S transition through regulating E2F by at least two mechanisms. First, by directly binding to E2F and preventing it from functioning as a transcriptional activator and second, by forming stable repressor complexes with E2F on cell cycle regulated promoters. The RB/E2F complexes are also known to directly interact with chromatin modeling enzymes, including SWI/SNF, histone acetyl transferases, and histone deacetylases, thereby regulating chromatin structure and gene activation/repression (Macaluso et al., 2006).

The RB proteins are regulated through sequential phosphorylation by CDKs (Weinberg, 1995). As cells pass through the R-point RB is phosphorylated by CDK4/6-cyclin D and then by CDK2-cyclin E on overlapping and distinct serine/threonine sites. Once RB is hyperphosphorylated, E2F becomes activated and promotes the transcription of genes required for DNA synthesis (Figure 1.1). Therefore, RB oscillates between a hyperphosphorylated and hypophosphorylated state as the cell progresses through the different cell cycle phases to control the activity of E2F (Harbour and Dean, 2000). Research in this area has therefore revealed that RB/E2F has an essential role in the regulation of the G1/S transition and is regulated by CDK-cyclin complexes in most cell types. As we will describe below, this is not the case for pluripotent stem cells.

## **GENERAL PROPERTIES OF PLURIPOTENT STEM CELLS**

Research over the past four decades has indicated that pluripotent stem cells can come in a variety of types. All of these types, however, satisfy the essential definition of a pluripotent stem cell, i.e.: having the ability to (1) undergo self-renewal and (2) multi-lineage differentiation. The original pluripotent stem cell that was described was an embryonal carcinoma cell type (Yu and Thomson, 2008) (see chapter 'History of stem cells'). These cells were derived from teratocarcinomas in the 1960s and despite their karyotypically abnormal status, they retained the ability to self-renew on feeder layers of mouse embryonic fibroblasts or differentiate when removed from the feeders. These cells provided the first evidence for the existence of



**Figure 1.1 Cell cycle control of differentiated/somatic cells and pluripotent stem cells (A)** Rb phosphorylation and inactivation by CDK-cyclin complexes regulates progression through the R-point, and entry into S phase in somatic or differentiated cells. **(B)** Mouse pluripotent stem cells lack a R-point and Rb is in a hyper-phosphorylated state throughout the cell cycle. Additionally, the CDK2-cyclin E complex is constitutively active throughout the cell cycle and E2F-transcriptional regulation is cell cycle independent.

pluripotent stem cells in culture. A major breakthrough, however, occurred in 1981 with the first works describing the derivation of mouse embryonic stem cells (mESCs) (Evans and Kaufman, 1981; Martin, 1981). Since then, numerous other pluripotent stem cell types have been described, including pluripotent embryonic germ cells (Matsui et al., 1992; Resnick et al., 1992), human embryonic stem cells (hESCs) (Thomson et al., 1998), epiblast stem cells (EpiSCs), which appear to be the mouse counterparts to hESCs (Brons et al., 2007; Tesar et al., 2007), and, most recently, induced pluripotent stem cells (iPSCs) generated by reprogramming somatic cells (Takahashi and Yamanaka, 2006). Reports describing murine iPSCs by Yamanaka were soon



followed by additional reports from the Yamanaka and Thomson labs for human iPSCs (Takahashi et al., 2007; Yu et al., 2007). Quite clearly, iPSCs hold great promise not only for disease therapies, but also for modeling diseases in cell culture.

Regardless of the type of pluripotent stem cell, self-renewal is maintained through a complex balance of extrinsic and intrinsic cues (Ng and Surani, 2011). Extrinsic cues generally rely on growth factors, such as LIF and BMP4 for mouse ESCs/iPSCs or TGFB1/Activin A, insulin/IGF-1 and FGF2/herregulin for human ESCs/iPSCs, to activate intracellular signaling cascades to promote self-renewal and block differentiation (Ohtsuka and Dalton, 2008; Singh et al., 2012). The core transcription factors Oct4, Sox2 and Nanog are central to sustaining stem cell pluripotency through activation of gene transcription while repressing genes important for lineage specification (Boyer et al., 2005). Recent studies indicate that core pluripotency factors also play a role in the initiation of differentiation, although a more thorough examination is still needed to fully understand its mechanism (Teo et al., 2011; Thomson et al., 2011; Yu et al., 2011). Most importantly, the extrinsic and intrinsic networks must cooperate to block differentiation to the three primary germ layers—mesoderm, endoderm and ectoderm—and extra-embryonic lineages, such as the primitive endoderm and trophectoderm (Ng and Surani, 2011). Furthermore, the extrinsic and intrinsic cues must promote the self-renewal of pluripotent stem cells by maintaining symmetrical cell division and promoting proliferation through the continuance of the cell cycle, without exit into G0.

## **THE PLURIPOTENT CELL CYCLE**

In the preceding section we described the regulation of the cell cycle as it generally applies for cell lines in culture. This is further representative of cell cycle regulation for eukaryotic somatic cells, which is generally conserved from yeast to mammals. However, pluripotent cells are atypical in many features, and this is especially evident when examining the cell cycle (Singh and Dalton, 2009; White and Dalton, 2005). In the following sections we will describe the unique features of the cell cycle of pluripotent stem cells, our current understanding of cell cycle regulation, and how this may pertain to the maintenance of pluripotency.

### **The Unique Cell Cycle Structure of Pluripotent Cells**

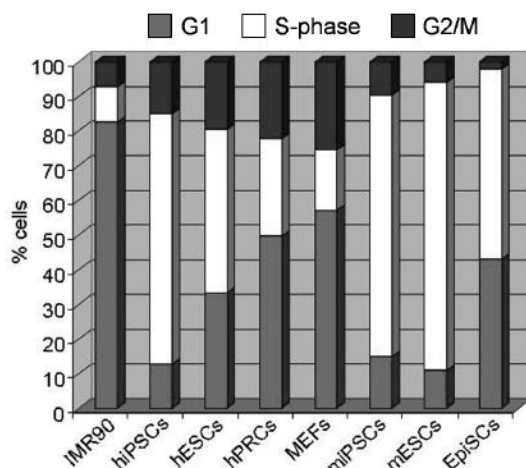
The cell cycle structure of pluripotent cells is distinct from that of somatic cells. For example, the cell cycle of pluripotent cells in the *Xenopus* pre-gastrula embryo consists only of alternative phases of chromosome

duplication (S phase) and cell division (M), without any gap (G1 or G2) phases (Murray and Kirschner, 1989). These observations can also be extended to drosophila and zebrafish. The purpose for this unique cell cycle structure is to promote the rapid cell divisions and cell numbers necessary for the early stages of embryonic development (Edgar and Lehner, 1996; Yarden and Geiger, 1996). Furthermore, since these non-mammalian species rely on pools of maternal RNA and proteins to perpetuate the cell cycle, zygotic transcription in G1 and G2 phases are unnecessary in early embryonic development.

In mammals, however, gap phases (G1 and G2) are present in the pluripotent cells of pre-implantation stage embryos but tend to be much shorter than in somatic cells (Lawson et al., 1991). Unlike *Xenopus*, pluripotent cells in mammals require zygotic transcription and translation and do not rely on maternal determinants to provide the factors required for DNA synthesis and subsequent cell division, which therefore explains the requirement for gap phases in mammalian cells. Another difference is that mammalian embryos increase in volume during early stages of development in contrast, for example, to *Xenopus* that maintains embryonic volume through cleavage divisions (Lawson et al., 1991; Snow, 1977). Analysis of the cell cycle of mammalian pluripotent stem cells has also demonstrated that they spend the majority of their time in S phase (60–80%), although the actual length of time it take for a pluripotent cell to proceed through S phase is equivalent to that of a somatic cell. During embryonic development as pluripotent cells differentiate, a re-structuring of the cell cycle profile takes place where by G1 is lengthened and the percentage of cells in S phase is reduced (Lawson et al., 1991). Nevertheless, the length of S phases is approximately the same.

For the most part, pluripotent stem cells in culture maintain the same cell cycle characteristics of pluripotent cells of the embryo (Singh and Dalton, 2009; White and Dalton, 2005). That is, they retain a shortened G1 with a high percentage of S-phase cells (Figure 1.2). Furthermore, as these cells are differentiated in culture, the cell cycle profile restructures to that of a somatic cell (G1 is lengthened and the percentage of S-phase cells is reduced). This appears to be the case for all pluripotent stem cells, including mESCs, hESCs, non-human primate ESCs, embryonic germ cells, EpiSCs, and iPSCs. The length of the cell cycle is different between mouse and human ESCs. On average, mESCs divide every 8–10 hours, while hESCs undergo a cell division after ca. 16 hours (White and Dalton, 2005; Becker et al., 2006). However, we should add that the population doubling times for hESCs are generally longer (ca. 25 hours) because apoptosis occurs during culture even though its underlying mechanisms are still unknown.

The purpose of the unique cell cycle structure of pluripotent stem cells is unclear. However, there may be a few reasons why this is important to



**Figure 1.2 Cell cycle profiles of differentiated/somatic cells and pluripotent stem cells.** DNA staining was used to analyze the cell cycle profiles of various cell types using flow cytometry. Percentage of cells in each cell cycle phase is mentioned for G1, S and G2/M. Human lung fibroblasts (IMR90), hiPSC, hESCs, human partially reprogrammed cells (hPRCs), mouse embryonic fibroblasts (MEFs), miPSC, mESCs, and EpiSCs are compared.

pluripotency (Singh and Dalton, 2009). The first is size control. Unlike many somatic cells, pluripotent cells tend to be much smaller and have a larger nuclear to cytoplasmic ratio. G1 is known to be a critical time for a cell in dictating cell size and the shortened G1 may therefore be important to keep a reduced cell size, conferring a large nuclear to cytoplasmic ratio (Saucedo and Edgar, 2002). The reason for the large nuclear to cytoplasmic volume of pluripotent cells compared to somatic cells is also unclear, but it may be representative of the chromatin structure of pluripotent cells, which is largely euchromatic (Herrera et al., 1996). Importantly, many genes needed for lineage specification are bivalent and are essentially in a state awaiting activation, such that they can specify differentiation (Bernstein et al., 2006). The chromatin structure of these genes therefore necessitates a largely open configuration.

Another possibility of why pluripotent cells maintain a shortened G1 is to prevent unwarranted differentiation (Singh and Dalton, 2009). Early studies have shown that embryonal carcinoma cells may specifically induce differentiation from the G1 phase of the cell cycle (Jonk et al., 1992; Mummery et al., 1987). Whether this translates to all pluripotent cells is still unknown. Nonetheless, this may suggest that cells in G1 may be more responsive to differentiation-inducing cues, such as particular growth factors or signaling pathways, Activin A, BMP, WNT or MAPK signaling, for example, that promote differentiation. Alternatively, S phase may be more refractory to differentiation inducing signals and may explain why

pluripotent cells spend a larger part of their cell cycle in this phase. However, much of this is currently speculative and more experiments are needed to address these questions.

## **Cell Cycle Control of Pluripotent Stem Cells**

The control mechanisms that regulate the cell cycle of pluripotent stem cells are considerably different from somatic cells. In mouse ESCs for example, the expression of many cyclins tends not to oscillate with the cell cycle, leading to continuous and elevated CDK activity (Stead et al., 2002b). This is especially the case for CDK2-cyclin E, which regulates the transition from G1 to S phase. One exception to this is cyclin B, which is needed for mitotic regulation at the G2/M border, and whose expression increases as cells transit from S to G2/M phase. In addition to the precocious activity of other CDK complexes, most CDKIs are not expressed or unable to block CDK activity (Faast et al., 2004; Stead et al., 2002b). These high levels of CDK activity permit rapid cell divisions during embryonic development and in ESCs cultures. During differentiation of ESCs, a decline of total CDK activity occurs in addition to the acquisition of CDK activity that is cell cycle dependent. CDKIs also become expressed during differentiation to regulate the cell cycle. Altogether, these observations are consistent with the slowing of the cell division rate of differentiated cells.

Furthermore, in mESCs RB exists in a hyperphosphorylated (inactive) state throughout the cell cycle and consequently, target genes under the regulation of E2F are not regulated in a cell cycle-dependent manner (White et al., 2005). Instead, E2F target genes are constitutively transcribed (Figure 1.1). In mESCs the triple knockout of RB family members leads to an inability to undergo normal differentiation to the primary germ layers while having no effect on their proliferation or self-renewal capabilities. This indicates a tight coupling of cell cycle control and differentiation (White and Dalton, 2005). Rb triple knockout mouse embryonic fibroblasts exhibit accelerated proliferation and a shortened G1, thus essentially taking on a mESC cell cycle phenotype (Dannenberg et al., 2000; Sage et al., 2000). Upon differentiation of mESCs, such as by LIF withdrawal, RB phosphorylation and E2F-regulated transcripts become cell cycle regulated (White et al., 2005). Importantly, the lack of Rb activity and E2F-regulated transcription during ESC self-renewal is indicative of the lack of R-point control and suggests an absence of mitogenic growth control. This suggests that mESCs divide in a cell autonomous manner, similar to that of tumor cells.

While hESCs appear to have cell cycle characteristics similar to mESCs, their regulation of the cell cycle have some differences. The expression of cyclins, for example, does appear to oscillate with the cell cycle, albeit to a lesser degree than in somatic cells (Neganova et al., 2009). This also appears

to be true for the expression of CDKs at the RNA level. At the protein levels, however, their expression is constitutive. Similar to mESCs, all RB family members are expressed in hESCs. But unlike in the mouse, where they are predominantly hyperphosphorylated and inactive, RB in hESCs appears to have both hyper- and hypo-phosphorylated forms, indicating differences in CDK activity and potentially, E2F regulation. This is especially true during the G1 phase of the cell cycle, where RB exists mostly in the hypophosphorylated form (Neganova et al., 2009). This would then indicate that, to some degree, RB and E2F targets are cell cycle regulated in hESCs. Similar to mESCs, hESCs express very low levels the CIP/KIP and INK4 CDKI families. Interestingly, however, overexpression of the CDKIs in hESCs appears to promote differentiation, but not necessarily cell cycle arrest as it does in somatic cell types (Ruiz et al., 2011).

Recent work has also found that proliferation and cell cycle regulators also play integral parts during the reprogramming process of somatic cells to iPSCs. For example, p53 (TP53) and INK4 have been found to serve as barriers to reprogramming (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). By deleting these genes, proliferation was markedly increased along with reprogramming efficiency. However, a consequence of these deletions was a lack of genomic stability in the reprogrammed cells. These data suggest that reprogramming efficiencies are affected by the expression levels of cell cycle regulators and that proliferation potential is a key determinant of a cells ability to be reprogrammed. Consistent with this, knockdown of Rb appears to increase the reprogramming efficiency, while overexpression of the CDKIs of the CIP/KIP or INK4 family decreases reprogramming efficiency (Ruiz et al., 2011).

## **The Role of Pluripotency Factors in Cell Cycle Control**

As described in the Introduction, pluripotency factors such as Oct4, Sox2 and Nanog, play critical roles in maintenance of stem cell self-renewal. In addition to these factors, Myc is also considered to play an important role in stem cell self-renewal. Several studies have now emerged demonstrating that these critical stem cell factors regulate the cell cycle. For example, Nanog appears to regulate the expression of CDK6 and CDC25A in hESCs (Zhang et al., 2009). CDK6 and CDC25A appear to regulate the length of S phase and the G1/S transition, as knockdown of these factors led to an accumulation of cells in G1. Other studies have indicated that Oct4 and Sox2 regulate miR-302, which impacts upon cyclin D1 expression in hESCs (Card et al., 2008) (see below).

At the heart of the self-renewal factors that impact upon cell cycle regulation is Myc. Myc has been shown to have a wide range of roles in the regulation and maintenance of stem cell pluripotency, including repressing primitive endoderm differentiation (Smith et al., 2010), regulating transcriptional pause-release (Rahl et al., 2010), metabolic control (Sloan and Ayer, 2010; Smith et al., 2011), and regulating epigenetic status (Lin et al., 2009). Perhaps one of the most essential roles of Myc is also to maintain the stem cell state by controlling cell cycle regulatory factors and proliferation (Singh and Dalton, 2009). While Myc is clearly expressed and involved in self-renewal of mouse and human pluripotent stem cells, its function in these cell types might be as variable as the mechanisms that regulate the cell cycle. This may be most evident from simple overexpression experiments. For example, Myc overexpression in mESCs promotes self-renewal and allows for the maintenance of stem cells in the absence of LIF (Cartwright et al., 2005). Conversely, its overexpression in hESCs has been found to promote apoptosis and differentiation (Sumi et al., 2007). Furthermore, LIF/Stat3 signaling regulates Myc expression in the mESCs, but most likely not in hESCs, since LIF signaling is not needed to maintain human pluripotent stem cells (Sumi et al., 2004). Additionally, while Myc expression levels appear to be relatively constant in mouse ESCs, they appear to be cell cycle regulated in human ESCs, peaking in G1 (Neganova et al., 2009). Deletion of *c-* and *n-Myc* in mouse pluripotent stem cells, like *nanog* deletion, results in the spontaneous differentiation to primitive endoderm, but whether this is also the case in human ESCs is still unresolved (Smith et al., 2010). Additionally, *c-* and *n-Myc* deletion in mouse stem cells results in the restructuring of the cell cycle, such that it resembles a differentiated somatic cell. This can be explained in part by the role of Myc in the regulation of the miR-17/92 locus and its targets like RB2 (see below).

As human pluripotent stem cells appear to have regulation similar to somatic cells, at least in part, we can draw on our knowledge of the function of Myc in these cell types for a role for Myc in the cell cycle control of human pluripotent stem cells. Like the RB/E2F complexes, Myc has a particularly important role at the G1/S transition. Loss of Myc leads to a lengthening of G1 (Shichiri et al., 1993), while overexpression of Myc leads to a shortening of G1 (Karn et al., 1989). Myc is known to regulate the expression of a wide array of genes important for the G1/S transition including cyclin D1, cyclin D2, cyclin E, CDC25A, E2F2 and E2F3 (White and Dalton, 2005). Perhaps, most important of these is Myc's role in maintaining a high cyclin E-CDK2 activity to promote the G1/S transition (Stead et al., 2002b). Equally important to this is Myc's ability to promote the sequestration and degradation of CDKIs (p27 and p21) through the regulation of cyclin D1 and D2 expression (Mateyak et al., 1999). The ultimate result of these effects is the shortening of G1 and a rapid progression through the cell cycle.

Furthermore, studies have shown that the deletion of *c-myc* in fibroblasts leads to a lengthening of both G1 and G2 (Mateyak et al., 1999). This suggests that Myc may have multiple roles in the cell cycle regulation beyond that of the G1/S transition.

## **The Role of miRNAs in Cell Cycle Control**

Micro-RNAs (miRNAs) have recently emerged as critical regulators of a number of processes, including embryonic development and differentiation, tissue homeostasis, and cell cycle control (Wang and Blelloch, 2009). MiRNAs are initially expressed as long transcripts that undergo two cleavage events resulting in a short RNA of approximately 22 nucleotides. The initial cleavage event occurs in the nucleus by the drosha/DGRC8 complex resulting in a pre-miRNA of ca. 70 nucleotides, while the second cleavage event occurs in the cytoplasm by the dicer complex and results in the mature miRNA. The primary function of miRNAs is to repress the translation of mRNAs by binding to the UTR (generally 3'-end).

Profiling of human and mouse ESCs has identified several families of miRNAs that are expressed in pluripotent cells (Houbaviy et al., 2003). As a single miRNA may have numerous targets, the function of these miRNAs in the stem cells may be widespread, from blocking differentiation factors to promoting self-renewal and proliferation. Further evidence for the functional importance of miRNAs has come from knockout experiments of *Dicer* and *Dgcr8* in mESCs. Deletion of *Dicer* or *Dgcr8* causes proliferation and differentiation defects in pluripotent stem cells (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2008b; Wang et al., 2007). Of particular interest *Dicer* and *Dgcr8* knockouts show an accumulation of cells in G1. Further investigations using the *Dgcr8* knockout model have identified miRNA that regulate the cell cycle and were dubbed *Embryonic Stem Cell enriched Cell Cycle-regulating* miRNAs. These include miRNA clusters miRNA-290, miRNA-302, and miRNA-17/20/106. In particular, miRNAs of these families were found to repress several targets including P21, RB-like 2 and LATS2, and thereby regulate the G1/S transition (Wang et al., 2008b). Interestingly, in hESCs miRNA-302 is expressed at very high levels and is regulated by Oct4/Sox2 (Card et al., 2008).

The miRNA-17/92 locus has also been found to be particularly important in mESCs and widely expressed (Houbaviy et al., 2003). This cluster is also down-regulated upon the differentiation of mESCs. This miRNA cluster is also widely associated with numerous tumors and cancers and is also known as *Oncomir-1* due to its oncogenic properties. This cluster is known to be regulated by Myc in tumors and strongly promote proliferation. A number of cell cycle targets are regulated by this locus including *E2f1*, *cyclin D1*, *P21*, and *Rb-like 2* (Fontana et al., 2008; O'Donnell et al., 2005; Smith

et al., 2010; Wang et al., 2008a; Yu et al., 2008). The miRNA-17/92 cluster therefore has direct role in maintaining the cell cycle, control at the G1/S transition and proliferation of mESCs. The extent to which this cluster is important for hESCs still needs to be examined.

## **CONCLUSIONS AND FUTURE PERSPECTIVES**

In the previous sections we have described how the maintenance of pluripotency is intimately linked with the cell cycle. While much of the mechanisms that regulate the cell cycle for mouse pluripotent stem cells have been elucidated, the mechanisms governing control of the cell cycle for human pluripotent stem cells still warrants further investigation. However, it is clear that despite the similarity in cell cycle structures of human and mouse pluripotent stem cells—that being, a short G1 and large percentage of S-phase cells—regulation in these two species is quite different (Neganova and Lako, 2008; Singh and Dalton, 2009), which several studies have demonstrated to be due to a different developmental stage. For example mESCs are representative of the early inner cell mass of a pre-implantation blastocyst, while hESCs are representative of the epiblast cells (Chenoweth et al., 2010). Therefore, it will be interesting to examine the cell cycle mechanisms that control mouse EpiSCs or early primitive ectoderm cells (Rathjen et al., 1999), which are isolated from a developmental stage closer to hESCs. Should these mouse cells have control mechanisms similar to hESCs, the conversion of mESCs to EpiSCs would be a useful model to examine the changes in cell cycle control that occur during pre-implantation blastocyst development.

The role of mitogens in control of the cell cycle of human pluripotent stem cells is also largely unclear. While mitogens do not appear to drive the cell cycle of mouse pluripotent stem cells (White and Dalton, 2005), they may have a role for human pluripotent stem cells. Of interest here is the wide array of differences in the growth factors and signaling networks that maintain pluripotency between human and mouse embryonic stem cells. For example, growth factors such as FGF2, heregulin, TGF- $\beta$ , and activin A are important to promote the self-renewal and growth of human pluripotent stem cells (Ohtsuka and Dalton, 2008; Singh et al., 2012), but these are not as important to maintain mouse pluripotent stem cells, where LIF and BMP4 appear to be the primary regulators (Ng and Surani, 2011). Whether hESCs can enter G0 following growth factor withdrawal is also an unanswered question. But since these cells appear to have a cell cycle regulation synonymous with somatic cells, at least in some aspects, this seems plausible.

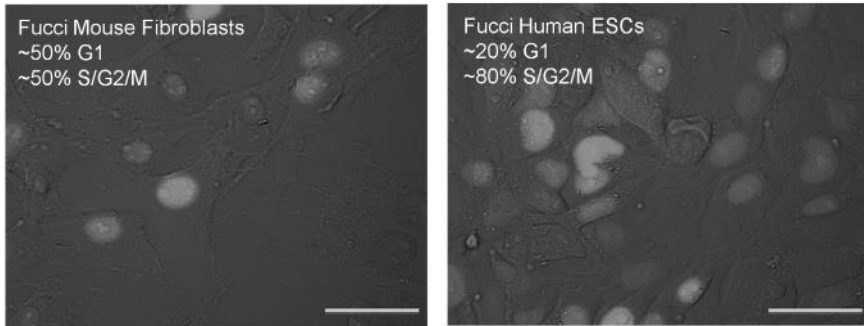
One of the most intriguing questions yet to be addressed is whether the unique cell cycle structure of pluripotent stem cells is a requirement for the maintenance of pluripotency. We fully expect, however, that imposing a



pluripotent cell cycle structure is not sufficient to promote pluripotency. For example Rb triple-knockout fibroblasts have a cell cycle structure similar to pluripotent cells, but are not capable of multi-lineage differentiation and cannot be described as pluripotent (Dannenberg et al., 2000; Sage et al., 2000). We should also add that the length of time required for progression through the cell cycle is also not a requirement for pluripotency. Addition of cell cycle inhibitors, for example, can lengthen the cell cycle while having no measurable impact upon differentiation potential (Stead et al., 2002a). This therefore rules out the hypothesis that rapid cell divisions are a requirement for pluripotency. Another intriguing question is whether the restructuring of the cell cycle (lengthening of G1 and a reduction in the percentage of S-phase cells) is a requirement for differentiation. We are unaware of any studies that directly address this question, most likely due to the difficulty in designing an experiment to test this.

One of the largest challenges in addressing these questions and examining the mechanistic control of the cell cycle has been the weaknesses in methodologies. Since mitotic shake-off experiments are difficult to routinely employ (because mESCs grow as domed-shaped colonies) most studies with pluripotent cells have relied on chemical inhibitors to synchronize cells by causing a cell cycle block. Removal of drug then allows cells to resume through the cell cycle in a synchronous manner. There are several caveats to these experiments, however. First, cell cycle inhibitors can be toxic and have undesired effects on the cells, such as cell death. Second, removal of the cell cycle inhibitor does not always result in complete release of the cells into the next cell cycle phase. This can then lead to an unintended level of contaminants in the cell cycle analysis. Third, and perhaps most importantly, a chemical block of the cell cycle can lead to a build-up of phase-specific proteins that may result in an rapid or uncharacteristic continuance of the cell cycle, particularly in the first cycle after release, which would result in a biased analysis.

Fortunately, several new tools have emerged that may aid us to more reliably characterize the cell cycle. Specifically, the emergence of fluorescence reporters and time-lapse cell culture techniques has provided significant advances over previous methodologies. For example, recent studies addressing the cell cycle have utilized a histone-2B fused to a GFP reporter (Kanda et al., 1998) that allows the purification and analysis of live, unsynchronized cell populations for a specific cell cycle phase based on histone content. Other reporter systems, such as FUCCI for Fluorescent Ubiquitination-based Cell Cycle Indicator, utilize fluorescent reporters that are degraded in particular phases of the cell cycle (Sakaue-Sawano et al., 2008), and therefore allow the purification of synchronized cells (Figure 1.3). Last of all, new DNA-binding dyes have emerged which allow the isolation of phase-specific cell cycle populations directly based on DNA



**Figure 1.3 Pictures of live FUCCI cells.** Mouse fibroblasts (left) and human embryonic stem cells (right) expressing FUCCI reporters to identify G1/G0 (red) or S/G2/M (green) cells.

*Color image of this figure appears in the color plate section at the end of the book.*

content. These new techniques provide significant advantages over previous methods as they do not biochemically perturb cells and allow a more reliable investigation of the mechanisms controlling the pluripotent cell cycle.

In summary, the cell cycle of pluripotent stem cells has many unique properties from that of somatic cells. While some of the mechanistic questions regarding how these unique properties are regulated have been answered, many other questions with regards to the importance, functionality, and role of the cell cycle in pluripotency are still unanswered. Since pluripotent stem cells share many properties and cell cycle characteristics with tumor cells, and provide significant potential for cell-based therapies, further studies into the role of the pluripotent cell cycle is warranted. We expect that this will be an area of intense investigation for years to come.

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## ABBREVIATIONS

|        |                                                                       |
|--------|-----------------------------------------------------------------------|
| MYC    | myelocytomatosis oncogene                                             |
| CDK    | cyclin-dependent kinase                                               |
| RB     | retinoblastoma                                                        |
| CDKN1A | cyclin-dependent kinase inhibitor 1A; more often identified to as P21 |

|        |                                                                       |
|--------|-----------------------------------------------------------------------|
| CDKN2A | cyclin-dependent kinase inhibitor 2A; more often identified to as P16 |
| LIF    | leukemia inhibitory factor                                            |
| TGFB1  | transforming growth factor, beta 1                                    |
| TB53   | tumor protein p53; more often identified to as P53                    |
| CDC25A | cell division cycle 25 homolog A                                      |
| LATS2  | large tumor suppressor, homolog 2                                     |

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# Asymmetric Cell Divisions and Nuclear Migrations of Neural Progenitors: Two Mechanisms that Influence Neurogenesis

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## SUMMARY

During the cell cycle, neural progenitors must constantly integrate intrinsic as well as extrinsic signals that influence progenitor cell behavior and fate decisions. Asymmetric cell division, in mitosis, and interkinetic nuclear migration, during interphase, are two potential regulators of cell fate decisions during the life-time of a cell. In this chapter, these two features will be introduced and discussed.

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*List of abbreviations after the text.*



## INTRODUCTION

Adult brains result from the growth, differentiation and morphogenetic movements of a simpler tissue structure called neuroepithelium. The neuroepithelium is initially formed by a single layer of cells, called neuroepithelial cells, that stretch from the ventricle to the pial surface of the epithelium. The neuroepithelial cells divide at the apical or ventricular surface of neuroepithelium. After cell division the daughter cell nuclei undergo a stereotyped movement towards the basal surface called interkinetic nuclear migration (INM). If daughter cells choose to re-enter the cell cycle their nuclei move back to the apical surface in the transition from S to G2 phases and divide again; if they choose to abandon the cell cycle during interphase then cells and nuclei move to the pial surface and differentiate into neurons. The layer of differentiating neurons close to the pial surface of the neuroepithelium is called the mantle layer and their axonal and dendritic arborizations first extend in the marginal layer just inside the pial surface. In the zebrafish spinal cord and mammalian cortex it has been shown that some subpopulations of neural progenitors, distinct from neuroepithelial cells, divide in more basal locations; these are collectively called basal progenitors but may be subdivided into several subtypes, e.g., intermediate, subventricular and outer subventricular zone progenitors.

Several mechanisms have been proposed to regulate cell fate decisions and explain the cell diversity in the developing brain. In this chapter we will focus on the regulatory roles of asymmetric mode of division and INM in cell fate decisions and differentiation of neurons.

The asymmetric mode of division is considered a cell lineage intrinsic mechanism that is widely used to generate cell diversity in non-vertebrates and vertebrates systems. Asymmetric divisions always generate two daughter cells with distinct fates, for example two distinct neural progenitors, two distinct neurons, or one neural progenitor and one neuron. It is also believed that this mode of division is widely used by stem cells in the adult brain to promote self-renewal and differentiation, but evidence for this is still missing. There is also evidence in vertebrates that asymmetric divisions result from asymmetric inheritance of structures and molecules, however the strength of this evidence is variable and the mechanisms that regulate such processes are still unclear. Recent reports suggest that cell cycle regulators or asymmetric activation of Notch signaling pathway can be downstream of asymmetrically inherited subcellular structures and molecules. INM is strongly correlated with cell cycle progression and cell cycle length can also influence cell fates. Recent findings suggest that INM can influence cell fate decisions through exposing cells to gradients of Notch signaling through the epithelia.

With the recent advances of genetic manipulations and live imaging techniques, our current understanding of asymmetric divisions and INM comes mostly from examining three vertebrate systems, mouse cortex, zebrafish embryos and slices of chick neural tube. Although they are still emerging systems compared to the mammalian cortex, the zebrafish and chick studies show some advantages in resolving cell behaviours because their superior optical qualities allow live imaging at high resolution.

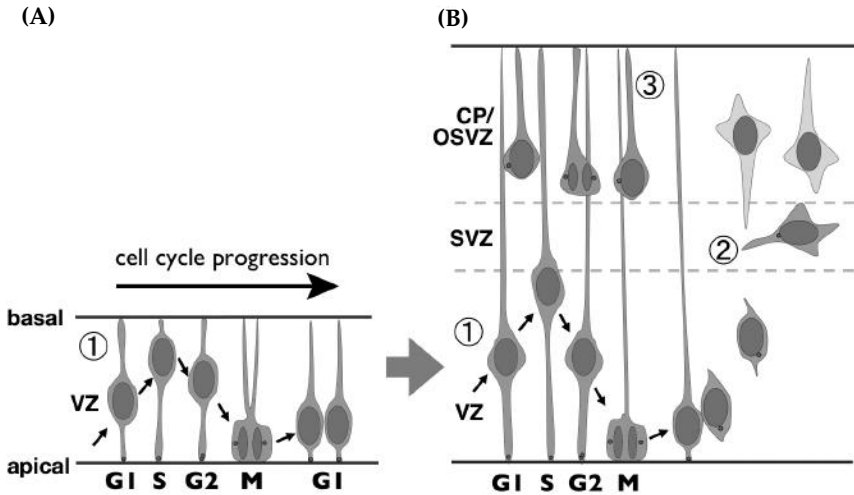
This chapter summarises our current understanding of asymmetric cell division, INM and their potential roles in vertebrate neurogenesis. We suggest that asymmetric cell divisions and INM act together to regulate neurogenesis by integrating signaling pathways involved in cell fate determination via cell lineage intrinsic and extrinsic mechanisms, respectively.

## **VERTEBRATE NEURAL PROGENITORS**

During vertebrate development, the generation of new born neurons (neurogenesis) within the central nervous system (CNS) begins in earnest after neural tube closure. The number of neurons in the vertebrate brain is superior to that of an invertebrates—for example, there are around 15 billion neurons in the human cortex. Once neurons are generated, they do not re-enter cell cycle. This implies that neuronal number is determined by the numbers and properties of neural progenitors and their lineages. Recent observations suggest several subtypes of neural progenitor cells with different capacities exist in the vertebrate CNS.

### **Neuroepithelial Cells**

The neuroepithelial cell (Figure 1A-1) is the first cell type that emerges in the developing vertebrate brain. It can directly or indirectly produce neurons and all subtypes of neural progenitor cells. Since other neural progenitors (see below) are not reported to generate neuroepithelial cell, this early cell type can be considered as the “stem” or mother of all other neural progenitor cells and neurons and glia produced during vertebrate CNS development. Neuroepithelial cells stretch from the ventricular (apical) surface to the pial (basal) surface. They are characterized by an apical and basal process on either side of the cell body and nucleus; the length of these processes depends on INM, that is, the phase of the cell cycle and the position of the nucleus. One process expresses apical proteins at the end of the cell that contacts the lumen of the neural tube. The other process reaches the basal lamina where components of the extracellular matrix, such as laminin and fibronectin are present. Those extensions are named ‘apical’ or ‘basal’



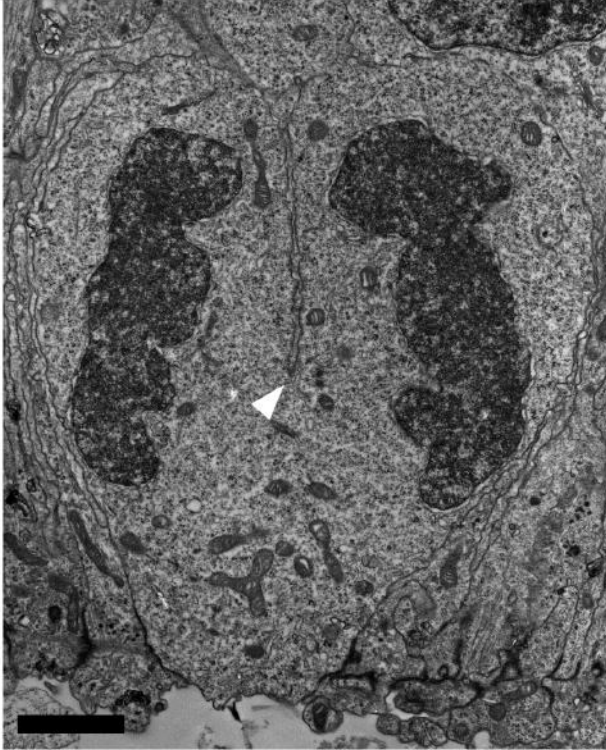
**Figure 1. Neural progenitors in the developing mammalian cortex** Drawings representing early (A) and mid/late (B) neurogenesis with neuroepithelial/radial glial cells (1), basal/intermediate (2) and OSVZ (3) progenitors. Nuclei of the former exhibit INM during the cell cycle (arrow). Each progenitor subtype can generate post-mitotic neurons (light blue). VZ=ventricular zone; SVZ=subventricular zone; OSVZ=outer subventricular zone; CP=cortical plate. Basal is up and apical down. Centrosomes are labeled in red.

*Color image of this figure appears in the color plate section at the end of the book.*

processes, respectively. Neuroepithelial cells are linked tightly together at their apical and basal ends by junctional complexes, such as adherens junctions and tight-junction at the apical end of the lateral cell membrane. The apical end feet of a neuroepithelial cell is also characterized by the localization of polarity proteins such as Par3, Par6 and aPKC and the stem cell marker CD133/prominin (Weigmann et al., 1997). Expression of these proteins gradually reduces as neurogenesis proceeds. The centrosome of neuroepithelial cells localize close to the apical membrane (Chenn et al., 1998) similar to other canonical epithelial cells (Reinsch and Karsenti, 1994). The most characteristic feature of neuroepithelial cell is that this cell shows INM, which will be discussed later.

Before the main period of neurogenesis starts, neuroepithelial cells increase in number, and the neuroepithelium grows. During this phase, the mode of cell division is largely “symmetric proliferative” (a progenitor divides to generate two more progenitors). After a certain period neurons begin to arise. Some will be generated by asymmetric division (a progenitor divides to generate a neuron as well as progenitor) while others are born from “symmetric differentiative” divisions (a progenitor divides to give rise to two neurons) (Lyons et al., 2003). The potential influence of cleavage plane and asymmetric inheritance will be discussed later but in the early

stages of the neural tube the cleavage plane is mostly close to perpendicular to the apical surface for all types of division (Figure 2; Kosodo et al., 2004; Götz and Huttner, 2005).



**Figure 2 A mitotic apical progenitor in the developing mouse brain.** Electron micrograph showing segregation of condensed chromatides. Arrowhead indicates the tip of the cleavage furrow bisecting the cell body. Basal is up and apical is down. Bar=1  $\mu$ m.

### ***Radial glial cell***

As neurons are born the neuroepithelium thickens and the component neuroepithelial cells transform into another progenitor subtype, and their name changes to “radial glial cell” (Figure 1B-1) (Götz and Huttner, 2005). Radial glial cells have common morphological features to neuroepithelial cells, such as presence of apical and basal processes that reach the ventricular and pial surfaces, respectively, and they exhibit INM. The apical and basal processes of radial glial cells are characteristically thinner than those of neuroepithelial cells. As neuroepithelial and radial glial cells have similar cellular features, they are often referred collectively to as “apical

progenitors” (Fish et al., 2008). Compared to neuroepithelial cell, radial glial cells are considered to display more differentiated features such as the expression of astroglial markers, for instance, GFAP (Götz and Huttner, 2005), however GFAP is also expressed in the earliest neuroepithelial cells of the zebrafish (Lyons et al., 2003; Tawk et al., 2007).

The cell cycle, especially G1-phase, of neuroepithelial and radial glial cells becomes significantly extended through neurogenesis (Takahashi et al., 1995). Radial glial cells can divide in symmetric proliferative mode, and asymmetric mode and symmetric differentiative modes. In asymmetric mode the divisions generate another radial glial cell together with either a neuron, or a basal progenitor or an OSVZ progenitor (Noctor et al., 2001; Miyata et al., 2001; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Shitamukai et al., 2011; Wang et al., 2011). In the later stage of brain development, radial glial cells produce the macroglia of the CNS –oligodendrocytes and astrocytes as well as ependymal cells and adult neural stem cells (constituents of ventricular wall in adult brain) (Gaiano et al., 2000; Kriegstein and Alvarez-Buylla, 2009).

### **Short Neural Precursor**

Short neural precursors have been identified in the embryonic mouse cortex in the VZ during mid- to late-(E13.6–16.5) neurogenesis (Gal et al., 2006). Short neural precursors have an apical process extending to the ventricular wall, like neuroepithelial and radial glial cells. In contrast to those cells, basal processes of short neural precursors do not reach to basal lamina and ends in the subventricular zone (SVZ) or intermediate zone (IZ). The cell cycle of short neural precursors, especially G1-phase, is significantly longer than that of radial glial cells in the same region of the developing brain (Stancik et al., 2010). Division of short neural precursors tends to produce neurons rather than basal progenitors (see below) (Stancik et al., 2010).

### **Basal Progenitor**

Basal progenitors (Figure 1B-2) are also referred to as “intermediate progenitors” or “non-surface dividing cells”. Basal progenitors have a distinct morphology compared to other neural progenitor cells, lacking apical or basal processes and connections. Basal progenitors start to appear at E11.5 and localize at the basal boundary of the VZ and SVZ in developing mouse cortex. They are generated by asymmetric divisions of radial glial cells and lose their apical process upon cell division or during interphase of the cell cycle (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). The population of basal progenitors is higher in the telencephalon

compared to midbrain or hindbrain in the mouse CNS (Haubensak et al., 2004). The cell cycle of basal progenitors is substantially longer than apical progenitors at the same developmental stage in mouse cortex (Arai et al., 2011). Basal progenitors can be recognized by specific expression of the transcription factor Tbr2 (Englund et al., 2005). Once Tbr2 is expressed, the cells do not show INM in the VZ, indicating that basal progenitors do not produce neuroepithelial and radial glial cells (Kawaguchi et al., 2008). Instead, a basal progenitor usually produces two neurons by symmetric differentiative division (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). In some cases, basal progenitors can generate another basal progenitor, but this type of division is minor (Noctor et al., 2004; Attardo et al., 2008). Cleavage plane orientation is relatively random compared to the apical surface (Attardo et al., 2008).

Basal neural progenitors have also been identified in the zebrafish spinal cord. Zebrafish basal progenitors divide close to the pial surface with random cleavages, some express Vsx1 and Vsx2 transcription factors and they undergo a terminal division to generate two distinct neurons, V2a and V2b interneurons (Kimura et al., 2008). The origin of zebrafish basal progenitors is not yet known and it is uncertain whether they share molecular properties with basal progenitors in the mouse cortex.

### **Outer Subventricular Zone (OSVZ) Progenitor**

The OSVZ progenitor is also referred to as an “outer radial glial (ORG) cell (Figure 1B-3) (Hansen et al., 2010)”. OSVZ progenitors were initially identified in the embryonic monkey brain (Smart et al., 2002) and subsequently characterized in gyrencephalic mammals such as human (Hansen et al., 2010) or ferret (Fietz et al., 2010; Reillo et al., 2011). This type of progenitor is now recognized in non-gyrencephalic primates (Kelava et al., 2012) and rodents (Shitamukai et al., 2011; Wang et al., 2011), suggesting a universal existence of this progenitor subtype among mammals. Morphologically, OSVZ progenitors have a basal process extending to the basal lamina similar to neuroepithelial and radial glial cells, however they either lack an apical process or their apical process does not reach the ventricular surface. OSVZ progenitors in the mammalian cortex express marker proteins similar to neuroepithelial and radial glial cells, such as Pax6 and Sox2, but not the Tbr2 transcription factor (Fietz et al., 2010; Hansen et al., 2010). Time-lapse imaging analysis demonstrated that this type of progenitor can produce more OSVZ progenitors, or basal progenitors and neurons by asymmetric divisions (Hansen et al., 2010). Cleavage plane orientation is near-random relative to the apical surface (Fietz et al., 2010).

## ASYMMETRIC DIVISION OF VERTEBRATE NEURAL PROGENITORS

This section gives an overview of the current evidence for mechanisms that might regulate asymmetrically fated divisions in the vertebrate neuroepithelium.

### Cleavage Plane Orientation

It is well established that during neurogenesis in *Drosophila*, the orientation of the mitotic spindle relative to the apicobasal axis is a critical determinant of the asymmetric segregation of fate determinants and therefore asymmetric daughter cell fates (Knoblich, 2008; Siller and Doe, 2009). This process was shown to be closely regulated by apical polarity complex (Par complex) and other proteins such as the adaptor protein Inscuteable (Insc), Pins, Galpha proteins and Mud that interact with microtubules and cell membrane, to precisely position the mitotic spindle in such a way that *Drosophila* neuroblasts always divide asymmetrically. There is evidence in vertebrates that Par complex, Insc, LGN and NuMa proteins (homologues of the *Drosophila* Pins and NuMa proteins) are also involved in mitotic spindle positioning. Armed with this knowledge the field of vertebrate neurogenesis has made strenuous efforts to establish whether similar mechanisms might also operate in the vertebrate neural tube. This has led to a heap of interesting but hard to interpret data, and even after 15 or so years it is fair to say that the importance and role of spindle orientation during vertebrate neurogenesis remains controversial.

One of the difficulties with these data is that the curved architecture of the brain ventricular surface makes an accurate analysis of the orientation of the mitotic spindle and cleavage plane rather difficult, resulting in a set of conflicting data (Haydar et al., 2003; Konno et al., 2008; Postiglione et al., 2011). A second problem is that there is no consensus as to how accurately these parameters need to be measured in order to make biological sense. Several studies simply divide the data into three categories—perpendicular, oblique and parallel cleavages (measured relative to the ventricular surface) while other studies argue that deviations in spindle orientation as small as 10° could well have functional relevance (Huttner and Brand, 1997; Kosodo et al., 2004). Despite these difficulties, a reasonable summary of the current data suggests that in the early neural tube (when few neurons are being generated and most progenitors divide symmetrically to give rise to two progenitors), cleavage planes tend to be close to perpendicular to the ventricular surface (Figure 2). Later, as more neurons are generated and progenitors often divide asymmetrically to give rise to two daughters with distinct fates, cleavage planes become more variable. Given this, it is

important to determine whether alteration of cleavage plane is a mechanism used by the progenitors of the neural tube to determine daughter cell fate. The answer to this question does not appear to be straight forward.

Several papers present data that suggest that a manipulation of cleavage plane can alter the dynamics of neurogenesis (Sanada and Tsai, 2005; Zigman et al., 2005; Fish et al., 2006; Postiglione et al., 2011) and the fate of daughter cells generated from divisions of vertebrate neural progenitors (Konno et al., 2008; Alexandre et al., 2010; Das and Storey, 2012). Although several different systems were analysed in these studies, the results are all consistent with the hypothesis that perpendicular cleavages favour symmetrically fated divisions and deviations from perpendicular favour asymmetrically fated divisions. However these data only support the conclusion that manipulation of the cleavage plane can alter cell fates in the vertebrate neuroepithelium, they do not directly show that this is a normal mechanism used by the neuroepithelium to regulate cell fates. In fact some data suggest that the symmetry of daughter cell fates may be unrelated to cleavage plane. For example, careful immunohistochemical analysis of fixed tissue showed symmetric or asymmetric inheritance of the apical membrane of progenitors does not correlate with cleavage plane (Kosodo et al., 2004). Similarly live-imaging of the apical domain in an intact neuroepithelium showed that this can be asymmetrically inherited by the two daughters that derive from divisions with perpendicular cleavage planes (Alexandre et al., 2010). Altogether these data suggest that although non-perpendicular cleavages do favour asymmetric daughter fates, asymmetric fate and asymmetric inheritance of sub-cellular domains do not necessarily depend upon deviations in cleavage plane.

## **Symmetric and Asymmetric Segregation of Fate Determinants**

The underlying assumption of all studies on the cleavage plane of neural progenitors is that this will be related to the symmetric or asymmetric inheritance of some factor(s) that can bias daughter cell fate. This is obviously a fundamental issue for this field of research but there is little direct evidence that asymmetric inheritance of any factors is important for asymmetrically fated divisions in the vertebrate neural tube. This is difficult evidence to obtain, because ideally it requires three things: first it requires direct visualisation that a component of the neural progenitor (such as a protein, or an RNA or a sub-cellular compartment) is indeed symmetrically or asymmetrically inherited by the daughter cells. Ideally this should be achieved by live imaging in an intact embryo neural tube. Second, it needs demonstration that the symmetry or asymmetry of inheritance is directly correlated with the fates of the daughters. This requires live imaging to the point where daughter cell fates can be validated. Third, it needs to be



shown that the observed symmetric or asymmetric inheritance is required for symmetry or asymmetry of daughter fates.

A small number of studies have achieved the first criterion (Chenn and McConnell, 1995; Miyata et al., 2001; Kosodo et al., 2004; Noctor et al., 2004; Konno et al., 2008; Bultje et al., 2009; Marthiens and ffrench-Constant, 2009; Alexandre et al., 2010; Shitamukai et al., 2011; Dong et al., 2012), five have achieved the second (Miyata et al., 2001; Noctor et al., 2004; Alexandre et al., 2010; Shitamukai et al., 2011; Dong et al., 2012) and only one to our knowledge is close to achieving the third (Dong et al., 2012). Live imaging in the zebrafish neural tube has determined that the Notch signalling regulator Mindbomb (Mib) is asymmetrically inherited during progenitor division. The daughter that inherits Mib is biased to become either a neuron or a progenitor with restricted neurogenic fate, while the daughter cell that does not inherit Mib becomes a self-renewing progenitor (Dong et al., 2012). Furthermore, asymmetric inheritance of Mib is dependent on the function of polarity protein Par3 (Dong et al., 2012) which is itself asymmetrically inherited during asymmetrically fated divisions (Alexandre et al., 2010).

The other examples that partially meet the three criteria for asymmetric inheritance are concerned with either the inheritance of the basal process of neural progenitors or with the inheritance of various components of the apical domain of the progenitors. Three studies (Noctor et al 2004; Alexandre et al., 2010; Shitamukai et al., 2011) used live imaging to reveal a correlation between the inheritance of the basal process of radial glial cells or neuroepithelial cells and a progenitor fate for the daughter. Another study showed that the basal process of radial glia is usually inherited by the neuronal daughter (Miyata et al., 2001)—the discrepancy with the other two studies may lie in the age of the tissue studied. At the apical end of progenitors, the apical membrane itself (Kosodo et al., 2004) and the apical polarity proteins aPKC and Par3 can be asymmetrically inherited (Kosodo et al., 2004; Marthiens and ffrench-Constant, 2009; Alexandre et al., 2010). Furthermore, the junctional domain expressing ZO1 can be asymmetrically inherited (Konno et al., 2008) but analysis of the N-Cadherin domain suggests that adherens junctions are split and not asymmetrically inherited along with more apical components (Marthiens and ffrench-Constant, 2009). With the exception of Par3 (Dong et al., 2012) the functional consequence of asymmetrically distributing these apical proteins or domains remains unknown.

## **THE ROLE OF POLARIZED PROTEINS AND SUBCELLULAR STRUCTURES DURING NEUROGENESIS**

As described above, epithelial organization and apico-basal polarity are key features of apical progenitor cells. Studies of cultured epithelial cells

identified that apico-basal polarity is formed and maintained by PDZ-domain containing apical protein complex, such as Par3/Par6/aPKC and Pals/Crb/Patj protein complexes (Margolis and Borg, 2005). These complexes localize to the distal end of the apical process, close to the apical membrane during interphase (Chenn et al., 1998). Upon entering the mitotic phase, some of these components, together with the apical membrane, may be inherited by only one of the daughter cells and potentially act as regulator(s) of asymmetric cell fate. This section discusses the function of polarity proteins in the regulation of neurogenesis and details some evidence they may influence asymmetric fates.

### **Par3/Par6/aPKC (the Par protein) Complex**

In *Drosophila* neuroblasts, the Par protein complex is at the top of a genetic hierarchy for specifying the polarity of epithelial cells and determination of asymmetric cell division (Johnson and Wodarz, 2003). In vertebrates, Par3, one of the Par complex proteins, is reported to associate with the adherens junctions of apical progenitor cells in mouse (Manabe et al., 2002), fish (von Trotha et al., 2006) and chick (Afonso and Henrique, 2006). Although Par3 inheritance and its role in cell fate choice of neural progenitors have been extensively investigated a clear consensus has yet to be established. It is reported that in asymmetrically dividing cells, Par3 is found unequally distributed together with apical membrane (Kosodo et al., 2004). In dissociated mouse neural progenitors, loss of Par3 function results in the premature exit from the progenitor state, while its over-expression promotes the generation of self-renewing progenitors (Costa et al., 2008). Another report demonstrates that Par3 surprisingly detaches from the apical end of the lateral membrane during mitosis, and shows asymmetric distribution in the cytoplasm during neurogenic division (Bultje et al., 2009). Interestingly, reduction of Par3 function increases the population of pairs of neurogenic daughter cells, while over-expression increases the number of pairs of progenitor cells. In both cases, daughter cells with an asymmetric cell fate are less common (Bultje et al., 2009).

However, recent studies that used live-imaging in zebrafish brain do not support this observation. In zebrafish asymmetric divisions, the daughter cell that inherits Par3 becomes the neuron while the other re-extends the apical process towards the apical surface after division and stays as a progenitor. The reduction of Par3 function results in the decrease of the proportion of asymmetric divisions and in the increase of proliferative divisions (Baye and Link, 2007; Alexandre et al., 2010; Dong et al., 2012). This work performed in zebrafish support the role of apical complex in promoting neurogenic fates but not proliferative fates as previously reported in the mouse cortex. What could be downstream of the asymmetric inheritance of the apical domain?

Experiments both in mouse and zebrafish suggest that Par3 may regulate Notch signaling pathway, however observations are again contradictory. In zebrafish it is suggested that Par3 inhibits Notch signaling (Dong et al., 2012) while in mouse studies it is suggested that Par3 promotes Notch signaling pathway activation (Bultje et al., 2009). In mouse knock-down of aPKC at later neurogenic stages does not have a significant effect on cortical neurogenesis (Imai et al., 2006), while early in zebrafish neurogenesis, reduction of aPKC function increased symmetric proliferative division and decreased neurogenic divisions (Baye and Link, 2007; Alexandre et al., 2010). It was shown in mouse that aPKC can be asymmetrically inherited, however it was never demonstrated that this correlates with asymmetric divisions (Marthiens and French-Constant, 2009).

### ***Pals/Crb3/Patj complex***

Pals1 has been reported to have critical functions in epithelial polarity and adherens junction assembly both in invertebrates and vertebrates (Margolis and Borg, 2005). Pals1 directly binds Par3/Par6/aPKC, thus linking both Par3/Par6/aPKC and Pals/Crb/Patj apical complex (Hurd et al., 2003). Remarkably, unlike its *Drosophila* homologue, stardust, Pals protein is required for normal corticogenesis in a dose-dependent manner as revealed by conditional knock-out analysis (Kim et al., 2010). Moreover, Pals1 is necessary and sufficient for self-renewal of apical neural progenitors, and its removal causes cell death possibly because Pals1 is essential in the transition from progenitor to neuron, at the very earliest stages of neuronal differentiation (Kim et al., 2010). Knock-down of Mals, a binding partner of Pals1, revealed its requirement for the maintenance of the apical localization of Pals1 in apical progenitors (Srinivasan et al., 2008). Loss of Mals function causes defects in the maintenance of apical polarity, and temporally affects brain development leading to a shorter cell cycle and increased production of post-mitotic neurons particularly in the early stage of corticogenesis (Srinivasan et al., 2008). Distribution and inheritance of Pals and Mals in mitotic apical progenitors are not known yet, and need to be examined to identify correlations for symmetric/asymmetric division.

### **Notch/Delta**

Several recent works in zebrafish and chick suggest that asymmetrically fated divisions are very likely to result from asymmetric activation of Notch signaling pathway. By using transgenes that report Notch activation (Zher4 and Ches5) it was revealed that in asymmetric divisions the basal daughter activates Notch signaling pathway and self-renewal while the

apical daughter undergoes differentiation (Das and Storey, 2012). How can the Notch signaling pathway be asymmetrically activated in asymmetric divisions? There is evidence that Notch signaling pathway components or modulators can be asymmetrically inherited and activate Notch pathway in one daughter cell (basal) but not in the sibling. The first evidence was given by early studies in mouse cortex that showed basal daughter cells resulting from parallel cleavages expressing Notch1a receptor. If neighbouring cells are expressing a Notch ligands and the basal daughter expresses Notch1a receptor it is very likely that the basal daughter will activate Notch signaling pathway and self-renew (Chenn and McConnell, 1995). Another recent finding in zebrafish revealed that Mindbomb, a Notch pathway modulator, is inherited by the neurogenic daughter in asymmetric divisions while the other progenitor daughter activates the expression of genes downstream of Notch activation (Dong et al., 2012). These observations therefore suggest that Notch signaling pathway can be directionally activated between the two sisters cells and remain cell lineage restricted, which still needs to be shown directly.

## **Numb**

Numb is a cytosolic protein, known to inhibit Notch and mediate cell-cell communication during metazoan development. Numb is asymmetrically localized to the differentiating daughter cell upon asymmetrically division of *Drosophila* neuroblasts (Knoblich, 2010). Mammalian Numb homologs are encoded by two genes, Numb and Numbl like (Zhong et al., 1996; Zhong et al., 1997). Knock-out of both Numb and Numbl like demonstrated their importance in brain development (Li et al., 2003; Petersen et al., 2004). Localization and role of Numb in mammalian neurogenesis have been investigated intensively, yet the outcomes differ among researchers. Early studies in the mouse cortex suggested that Numb localizes to the apical side of asymmetrically dividing cells (Zhong et al., 1996; Petersen et al., 2002), while in zebrafish expression of a Numb-GFP fusion construct presented a basolateral distribution in dividing progenitors (Alexandre et al., 2010). Unlike in *Drosophila*, there is little evidence that Numb and Numbl like regulate Notch in the vertebrate neuroepithelium suggesting that Numb function can vary between different systems or contexts.

By immuno-electron microscopy in the mammalian cortex, it has been shown that Numb in mitotic cells localizes to vesicles associated with adherens junctions. Moreover, both Numb and Numbl like are required for maintaining the polarized structure of radial glia and epithelial integrity by correctly targeting adherens junctions components (Rasin et al., 2007). Another report demonstrates that Acdb3, a protein that binds to the Golgi membrane protein Giantin, directly binds Numb. Acdb3 associates with the

Golgi apparatus during interphase, while diffusing to the cytosol after Golgi fragmentation during mitosis. Mis-expressing a cytosolic form of Acbd3 during interphase inhibited neurogenesis, suggesting that Numb signaling is regulated through changes in the subcellular distribution of Acbd3 (Zhou et al., 2007). The cellular distribution and inheritance of vertebrate Numb proteins thus remains controversial and requires further analysis.

## **Basal Process**

Among subtypes of neural progenitors, apical progenitors and OSVZ progenitors possess thin, long processes extending from their somata toward the basal lamina and referred to as “basal process” (see above). Previously, the basal process was believed to be lost during mitosis and then to re-grow after cell division. Time-lapse imaging in rat (Noctor et al., 2001), mouse (Miyata et al., 2001) and zebrafish (Das et al., 2003) revealed that the basal process is retained during cell division and it is always in contact with the basal lamina. This fact promoted research about its role for asymmetric cell division and whether asymmetric inheritance of the basal process might correlate to cell fate determination (Miyata et al., 2001; Fishell and Kriegstein, 2003; Wodarz and Huttner, 2003). Consistent with this, recent live imaging analyses demonstrated that apical (Alexandre et al., 2010; Shitamukai et al., 2011; Wang et al., 2011) or OSVZ (Fietz et al., 2010; Hansen et al., 2010) progenitors inheriting the basal process tends to self-renew while the sibling cell without basal process differentiates becoming a neuron or basal progenitors. During proliferation of apical progenitors, when symmetric divisions are dominant, the basal process tends to split prior to the cytokinesis in spite of its thin and long structure in the developing brain of mouse and zebrafish (Kosodo et al., 2008). These results strongly suggest a role of the basal process for fate decision of apical and OSVZ cells. Indeed, importance of the contact of the basal process to the basement membrane has been revealed by recent studies. In conditional knock-out mouse of integrin beta1 subunit gene, basal processes are detached from the meningeal basement membrane (Radakovits et al., 2009). Although cell proliferation or INM is normal, enhanced apoptosis is observed in the mutant brain. This effect is more evident during early phase of cortical development when proliferative, symmetric divisions are dominant (Radakovits et al., 2009).

If signaling for cell fate decisions is transmitted through the basal process, which molecules are involved? A recent study reported that the mRNA of cyclin D2, a positive regulator of G1 progression, accumulates in the basal process and is asymmetrically inherited by the basal daughter and not by the apical one (Tsunekawa et al., 2012). The inheritance of cyclin D2 by the basal daughter cell is in agreement with previous reports about

the role of basal process inheritance in self-renewing properties (Kosodo et al., 2008; Alexandre et al., 2010). Another possible candidate might be all-trans retinoic acid (atRA), which is secreted from embryonic meninges and requires Foxc1 for its action (Siegenthaler et al., 2009). In Foxc1 mutant mice, neural progenitors switching from symmetric proliferative division to asymmetric neurogenic division are reduced and this can be rescued by extra supply of atRA (Siegenthaler et al., 2009). Considering that the basal process may receive signals essential for proliferation or differentiation may lead to future studies aimed at identifying the existence of specific receptors on the process itself.

## **Centrosomes**

The centrosome consists of two microtubule-based cylinders called centrioles and it is the main microtubule-nucleating organelle in animal cells. It plays an essential role in mitotic spindle orientation, genome stability and ciliogenesis (Bornens, 2012). Centrosomes duplicate once per cell cycle by disengagement of the centriole pair at the end of mitosis. In S-phase, the new centrioles (called daughter centrioles) are assembled next to the “mother centrioles”. The two centrosomes formed by a pair of “mother” and “daughter” centrioles, position at the spindle poles during mitotic phase and each pair is inherited by one of the daughter cells. Considering this process of biogenesis of centrosomes, their inheritance is, per definition, asymmetric with regard to the age of each centriole.

Importantly, mother and daughter centrioles have structural and functional differences. The older centriole is longer than the younger and possesses distal and subdistal appendages that anchor microtubules and cell membrane. The distal appendages bear microtubule anchoring proteins such as ninein (Mogensen et al., 2000). Shorter daughter centrioles lack microtubule and cell membrane anchoring functions.

It was not until recently that the combination of photoconversion approaches and old and young centrosomal markers finally distinguished between young and old centrosomes in dividing *Drosophila* neuroblasts. The combination of these approaches revealed that the mother centrosome is in fact inherited by the ganglion mother cell and not by the self-renewing neuroblast, as thought for a long time (Conduit and Raff, 2010; Januschke et al., 2011). This result differed from what has been previously shown in other systems such as *Drosophila* male gonads (Yamashita et al., 2003) and developing mouse neocortex (Wang et al., 2009). In these systems it is reported that self-renewing stem cells and apical progenitors inherit the older centrosome, while the other daughter cell inherits the younger centrosome. Moreover, centriole maturation is lost when ninein is removed and the pool of apical neural progenitor is converted to cells expressing

neuronal markers (Wang et al., 2009). The molecular mechanism by which asymmetric centrosome inheritance can regulate cell fate is not described yet. One possibility is that, as more than 100 proteins localize in the centrosome matrix including centriole (Nigg and Raff, 2009), proteins crucial for fate-determination might differ between the two centrosomes. It should be also investigated how asymmetric centrosome inheritance can be reconciled with symmetric, proliferative division of apical progenitors prior to the initiation of asymmetric division (Fietz and Huttner, 2011).

## **INM OF VERTEBRATE NEURAL PROGENITOR CELLS**

Among the neural stem cells introduced earlier, the nuclei of apical progenitor cells, that is, neuroepithelial cells and radial glial cells, show a characteristic movement along their apicobasal axis during the cell cycle. Mitosis of apical progenitor cells takes place at the apical surface. After mitosis, nuclei migrate basally through the VZ in G1. Nuclei enter S at the basal region of the VZ, then they return apically for the next mitosis. This movement of nuclei is named “interkinetic nuclear migration” (INM) (Figure 1). INM was originally identified in the neural tube of developing vertebrate embryo (Sauer, 1935). After that, the phenomenon was revealed not only in neural tubes of vertebrates but also in several other pseudostratified epithelia, even in invertebrates such as in the wing disk of *Drosophila* and in the ectoderm of *Nematostella* (Table 1). From these observations, INM is considered as a “hallmark” of pseudostratified epithelia (Kosodo, 2012). Although INM exists in postnatal tissue, in most of cases, this is more common in embryonic stages (Table 1) where somatic progenitors are abundant. This fact raises the possibility that INM may act on the regulation of progenitor cell fate, that is, proliferation and differentiation.

How nuclear movement is controlled in accordance with cell cycle phases while preserving tissue integrity is a fascinating question. To clarify the regulation of INM, molecules involved for its progression have been investigated by drug treatments in the early studies and genetic manipulations more recently. Although some aspects of INM still need to be determined, many cell biological features have been characterized. In this section, the mechanisms that regulate INM will be discussed and the possible effects of INM on cell fate determination summarized.

### **Cell Cycle Regulation**

Nuclear movement of apical progenitors is tightly coupled to cell cycle progression during INM. From this, it has been proposed that cell cycle

**Table 1** Species and tissues in which INM is documented (adopted from Kosodo, *Cel Mol Life Sci* 2012).

| Species             | Tissue       | Proposed driving forces              | References                                  |
|---------------------|--------------|--------------------------------------|---------------------------------------------|
| Human               | Brain, other | -                                    | Fujita et al., 1960                         |
| Mouse               | Brain        | B to A dynein; A to B actomyosin     | Schenk et al., 2009                         |
|                     |              | B to A dynein; A to B displacement   | Kosodo et al., 2011                         |
|                     | Retina       | -                                    | Saito et al., 2003                          |
|                     | Liver bud    | -                                    | Bort et al., 2006                           |
|                     | Intestine    | -                                    | Grosse et al., 2011                         |
| Rat                 | Brain        | B to A dynein; A to B kinesin        | Tsai et al., 2010                           |
| Chick               | Brain        | Microtubule / actomyosin             | Langman et al., 1966 / Meisser et al., 1974 |
|                     | Retina       | -                                    | Pearson et al., 2005; Becker et al., 2007   |
| Zebrafish           | Hindbrain    | B to A actomyosin; A to B stochastic | Leung et al., 2011                          |
|                     | Retina       | B to A actomyosin; A to B stochastic | Norden et al., 2009                         |
| <i>Drosophila</i>   | Wing disc    | Actomyosin                           | Meyer et al., 2011                          |
| <i>Nematostella</i> | Ectoderm     | Microtubule / actomyosin             | Meyer et al., 2011; Nakanishi et al., 2011  |

A; apical, B; basal. Tissues are embryonic.



progression can be a driver of INM, or that nuclear positions can promote cell cycle progression. It has been shown that inhibition of INM by disrupting microtubules or using actomyosin inhibitors has no effect on cell cycle progression (Karfunkel, 1972; Messier and Auclair, 1974; Messier, 1978; Murciano et al., 2002; Gambello et al., 2003). On the other hand, cell cycle perturbation at G2/M by 5-azacytidine and S by cyclophosphamide resulted in the accumulation of nuclei in the apical region and the basal region, respectively (Ueno et al., 2006). These results were reproduced by using other drugs, that is, hydroxyurea, aphidicolin and RO-3306 for S and G2/M inhibition, respectively (Leung et al., 2011). By genetic manipulation, G1 phase arrest was achieved by over-expression of p18<sup>Ink4c</sup> (a cyclin-dependent kinase inhibitor) leading to basal accumulation of nuclei (Kosodo et al., 2011). Taken together, these results indicate that cell cycle progression likely regulates the activity of nuclear migration.

### **Microtubule, Centrosome and Related Motor Proteins**

To translocate nuclei, motor proteins generate actual forces by hydrolyzing ATP or GTP. For INM, two such cytoskeletal motor protein pathways are proposed; one is “microtubule/dynein or kinesin”, and the other is “actin/myosin II”. These motor proteins act like locomotives on a cytoskeletal railway. Microtubule rails have a polarity; indicated by the plus-end, the fast growing end, and the minus-end. The minus-end of microtubules is located on the centrosome, a microtubule-organizing center of animal cells. In epithelial cell, centrosome localizes just close to the apical surface (Reinsch and Karsenti, 1994), therefore minus-end to plus-end microtubule polarity is parallel to apical to basal epithelial polarity.

Several components of microtubule-based motor systems are known to be involved in INM. The Lis1 protein regulates dynein, a microtubule minus-end-directed motor protein, by a direct protein-protein interaction. Reduced protein levels of Lis1 causes the inhibition of basal to apical nuclear migration (Gambello et al., 2003; Tsai et al., 2005). Dynactin-1 (Del Bene et al., 2008) and NudC (Cappello et al., 2011) are also required for basal to apical nuclear migration. As dynein/dynactin/Lis1/NudC complex are co-immunoprecipitated in mammalian cells, the function of the protein complex as a motor on microtubule cytoskeleton appears to be essential for basal to apical nuclear migration. A mutant form of Lis1 causes lissencephaly, a developmental disorder of the human brain that results in an almost complete loss of cortical gyri (Wynshaw-Boris et al., 2010). The loss of Lis1 function also affects the migration of post-mitotic neurons as well as INM.

For apical-to-basal nuclear migration, Kif1a, a member of kinesin-3 family protein, may have an important role. Kif1a contains an N-terminal motor domain, and this type of kinesin transports cellular cargos toward the microtubule plus end. As indicated above, the microtubule plus end extends toward the basal side of neural progenitors. Kif1a knock-down showed defects in the basally directed nuclear migration, while apical migration is regulated by dynein motors (Tsai et al., 2010). How those dual motors for INM are co-ordinated with the cell-cycle phases needs to be examined.

Centrosomes are foci for microtubule filaments localization to the apical surface of apical progenitor, and centrosomal activity at the apical surface is considered to play an important role in INM. The ectopic position of the centrosome in Pax6 mutants caused aberrant INM (Tamai et al., 2007). Cep120, a centrosomal protein preferentially expressed in neural progenitors, and transforming acidic coiled-coil proteins (TACCs), another centrosomal protein that binds to Cep120, contribute to INM (Xie et al., 2007). It is also reported that Hook3, another centrosomal protein, acts as microtubule anchorage to the centrosome through the interaction with Pericentriolar Material 1 (PCM1) with each protein being needed for INM and neurogenesis (Ge et al., 2010).

The physical link between nuclear envelope and dynein complex is accomplished by Kash and Sun proteins. In the perinuclear space, Sun proteins interact with Kash proteins, which span the outer nuclear membrane. In the cytoplasm, Kash proteins interact with dynein to form a bridge between the microtubules and the inner part of the nucleus. Loss-of-functions of Sun1/2 or Kash proteins (i.e., Syne-1/2) impair INM, suggesting that the physical link between nuclear envelope and dynein complex is critical (Del Bene et al., 2008; Zhang et al., 2009; Yu et al., 2011).

Regulation of microtubule assembly during interphase has a significant effect on the efficiency of INM. Tpx2, a microtubule-associated protein, localizes to the microtubules in the apical process (but not in the basal process) of neural progenitors. The expression of Tpx2 is cell cycle dependent, and the protein accumulates in the apical process of cells in G2. By knocking down Tpx2, microtubule bundles in the apical process of G2 cells are loosened, and basal to apical nuclear migration is perturbed (Kosodo et al., 2011). Tpx2 function connects cell cycle phases and the organization of microtubule cytoskeleton required for INM.

## **Actin and Related Motor Proteins**

In addition to the microtubule cytoskeleton, the actin cytoskeleton is also considered to have a prominent role in INM. Studies using cytochalasin B, an inhibitor of actin polymerization, indicated that proper actin organization is required for INM (Messier and Auclair, 1974; Murciano et al., 2002), although

it has been unclear which phase of nuclear migration is regulated by actin organization. Recent molecular studies indicated two possibilities. One is that the actin cytoskeleton contracts on the apical side of nuclei together with interaction of the non-muscle motor protein myosin II, and nuclei are pushed toward the basal region in G1 (Schenk et al., 2009). The other is that the actomyosin system drives basal to apical nuclear migration by the G2-specific accumulation of myosin II at the basal side of the nucleus (Norden et al., 2009; Leung et al., 2011).

So far, both microtubule-dynein and actomyosin are proposed for INM, which might depend on the tissue and organism considered (Table 1). Possibly, several factors in INM such as cell shape, distance of nuclear migration, and evolutionary aspects may determine the balance between microtubule-dynein and actomyosin systems.

### **Small GTPases**

In addition to significant roles for microtubule and actin cytoskeletons, the functions of small GTPases need to be determined for proper INM, since these proteins are generally upstream of cytoskeletal organization. Indeed, loss-of-function form of Cdc42 (Cappello et al., 2006) or Rac (Minobe et al., 2009) disrupts INM. However the small GTPases are multi functional molecules, and their precise role is yet to be determined.

### **Apico-basal Polarity**

Apico-basal polarity of epithelial cells is established by conserved protein complexes, such as the Par polarity complex, Crumbs, and Scrib complexes (Martin-Belmonte and Perez-Moreno, 2012). The Par complex consists of Par3, Par6, and atypical Protein Kinase C (aPKC) that localize close to the apical surface and has an important role for INM. Conditional knock-out of aPKC $\lambda$ , one of two aPKC members in mouse, resulted in a retraction of the apical process of apical progenitor cells, disappearance of apical junctions, and mis-localisation of nuclei in S-phase due to aberrant INM in mouse and Zebrafish (Imai et al., 2006; Baye and Link, 2007). Knock-out of Aspp2, a binding partner of Par3, affects INM and lamination of the cortex and retina (Sottocornola et al., 2010). Taken together, these results indicate that loss-of-function of the Par complex induces the disruption of epithelial polarity, which results in the perturbation of INM.

Basal proteins as well as apical proteins may have a significant role in INM. Laminins are heterotrimeric glycoprotein complexes consisting of  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits, and are principal structural components of basement membranes of epithelial cells. Mutation in laminin  $\gamma$ 1 in *Medaka* embryos

causes abnormal mitotic patterns in the neuroepithelium, and nuclei exhibit abnormal and variable patterns of migration. Interestingly, the polarity and shape of apical progenitor cells are not affected in this mutant (Tsuda et al., 2010). This result indicates that laminin complex or basal lamina is important for INM. However, other report demonstrates lack of apparent defect in INM by knocking-out laminin  $\gamma$ 1III4 from mouse brain, although the interaction between basal process and the basal lamina is disrupted (Haubst et al., 2006). The role of the basal lamina and the basal process for INM needs further investigations.

## **Gap-junctions**

A gap junction is a specialized intercellular connection directly connecting the cytoplasm of two cells and characteristic of a multitude of animal cell-types and allows small molecules and ions to pass freely between cells. It has been proposed that neuronal precursors in the VZ are extensively coupled in radial columnar clusters via gap junctions (Lo Turco and Kriegstein, 1991) and that gap junctions are involved in the propagation of  $\text{Ca}^{2+}$  waves in the proliferative zone of the embryonic brain (Weissman et al., 2004). Interestingly,  $\text{Ca}^{2+}$  waves might affect the progression of INM. Spontaneous  $\text{Ca}^{2+}$  transients precede nuclear movements and inhibiting  $\text{Ca}^{2+}$  transients significantly impaired INM in the chick retina (Pearson et al., 2005). Blocking gap junctions or knocking-down Connexin 43, a subunit of the hemichannels of gap junctions, prevented basal to apical nuclear migration. This could be due to a cytoskeletal reorganisation resulting from phosphorylation of the small GTPase Cdc42 in apical progenitors resulting in impaired gap junction function (Liu et al., 2010).

## **Mechanical Factors**

Emerging evidence indicates that, in addition to molecular regulation, the physical displacement of nuclei in tissues can be a driving force for a particular direction of INM. This is because the pseudostratified tissue in which INM occurs is generally highly packed with nuclei. In such dense tissue, active nuclear movement in some cells can create a passive displacement of other cells to generate space. The possibility of the existence of this passive factor for basally directed nuclear movement is in fact mentioned in the first report of INM in 1935 (Sauer, 1935). Until recently, however, this passive factor has not been investigated carefully. One report proposed that active driving forces for basal-to-apical nuclear migration is generated by actomyosin (Norden et al., 2009) while another proposed the involvement of microtubule-dynein motor systems (Kosodo et al., 2011).

However, in both studies the apical-to-basal nuclear movement was mainly dependent on a passive displacement exerted by the active basal-to-apical migration of neighboring nuclei. This mechanical “knock-on” mechanism may be the best way to balance the position and density of many nuclei undergoing INM within the epithelium, rather than having to somehow balance the speed and directionality of opposing molecular mechanisms in many independent cells.

### **Affect on Fate of Progenitor Cells by Perturbation of INM**

In the neuroepithelium of the developing mammalian brain, nuclei can move more than 80  $\mu\text{m}$  in one cell cycle with speeds of around 1  $\mu\text{m}/\text{min}$  (Kosodo et al., 2011). The high density of apical progenitors all oscillating through the cell cycle generates a highly dynamic environment with nuclei potentially able to experience different molecular environments in different phases of INM. It is likely that both intracellular and intercellular signaling molecules controlling cell fates are present in particular apico-basal regions of the tissue and may thus regulate fate decisions in different phases of the cell cycle. In addition, impaired INM may induce abnormally biased localization of signaling molecules, which may result in defective regulation of neuroepithelial development. Indeed, some reports suggest that INM is required for the proper proliferation and differentiation of progenitor cells, especially in the development of central nervous system (reviewed by Kosodo, 2012).

### **Centrosomal Activity**

The involvement of centrosomal activity in the fate choice of neural stem cells has been deduced from human genetic studies. Mutations in genes coding for centrosomal proteins gave rise to severe defects in brain size and neuronal number (*microcephaly*) (Thornton and Woods, 2009; Kaindle et al., 2010). As centrosomes are key organelles for INM, brain defects in microcephalic patients might be a result of aberrant INM. The relationship among centrosomal activity, INM defects, and the reduced number of neurons has been investigated using model animals to examine the role of INM for the fate choice of the neural progenitors (Ge et al., 2010). Indeed, impaired centrosomal function in apical progenitor cells resulted in defective neurogenesis. Pericentriolar Material 1 (PCM1)-containing pericentriolar satellites were found at the apical surface of neuroepithelial cells in the developing neocortex. Hook3, that links centrosome and the

nucleus, is recruited to pericentriolar satellites through an interaction with Pcm1. Knocking-down Pcm1 or Hook3, or blocking the Hook3-Pcm1 interaction, affected INM increasing precocious neurogenesis and depleting the progenitor pool (Ge et al., 2010).

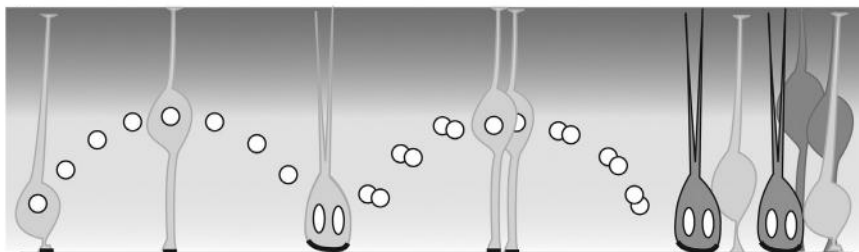
## **Gradient of Signaling Molecules**

During INM, the amplitude of nuclei oscillation differs among cells even in the same brain region. By using bromodeoxyuridine (BrdU) together with  $^3\text{H}$ -thymidine, it was shown that the extent and rate of apical to basal nuclear movement during G1 is variable and that nuclei do not change positions much in the basal region of the neuroepithelium during S-phase (Hayes and Nowakowski, 2000). Interestingly, some reports demonstrated that there are certain correlations between S position and cell fate of neural stem cells. In the zebrafish retina, time-lapse imaging revealed proliferative and neurogenic progenitors that reached different distances from the apical surface during S (Baye and Link, 2007). One possible scenario to generate different cell fate is a concentration gradient of morphogens along the epithelium's apico-basal axis such that nuclei might encounter different amounts of these factors during specific cell cycle phases during INM (Latasa et al., 2009). Consistent with this, molecules implicated in Notch signaling, whose activity can promote proliferation and cell cycle re-entry, showed heterogeneous distributions along the apico-basal axis and INM defects that bias nuclei to the basal side of the epithelium were suggested to reduce the exposure of progenitors to Notch activation leading to precocious neurogenesis in the zebrafish retina (Del Bene et al., 2008).

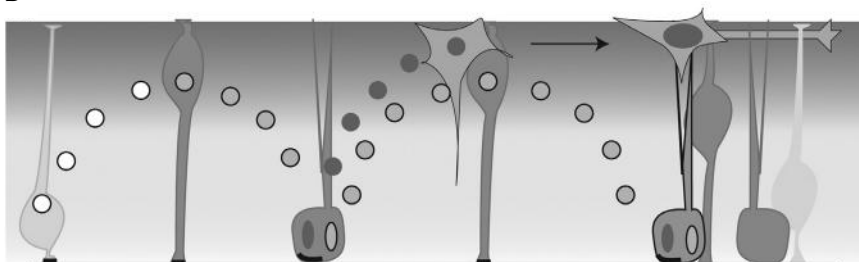
## **CONCLUSIONS AND FUTURE PERSPECTIVES**

An extensive literature on INM strongly suggest that this process is crucial for cell fate change of neural progenitors leading to models connecting it to asymmetric cell division and neurogenesis (Figure 3). This model suggests that nuclei position can contribute to commit neural progenitors to differentiation, while asymmetric divisions can determine which of the daughter cells becomes the neuron and which remains a progenitor. However, the understanding of the molecular mechanisms connecting cell cycle progression and cell fate change by INM as well as its connection to intracellular and environmental factors is still in its infancy and has yet to be fully determined.

**A**



**B**



(adapted from Baye and Link, 2007 and Del Bene et al, 2008)

**Figure 3 A Model representing the complementarity of INM and asymmetric cell division regulating neurogenesis** (A) Prior to a symmetric proliferative division, the cell nucleus does not reach the neurogenic region (orange/red) at the basal surface and therefore is less likely to generate neurons, as initially proposed by Baye and Link, 2007. During symmetric divisions, the apical domain (black; with inheritance correlating with symmetric cell division) is symmetrically inherited by the two daughter cells. (B) In neurogenic divisions, cells nuclei move further basally and reach a putative neurogenic region (orange/red). This probably contributes to committing the progenitor (dark grey) to produce at least one neuron (light blue) in the next cell division. The asymmetric inheritance of factors and structures, as illustrated by asymmetric inheritance of the apical domain (black) and basal process, promotes daughter cells to adopt asymmetric fates (for example, neuron and progenitor).

*Color image of this figure appears in the color plate section at the end of the book.*

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## ABBREVIATIONS

|     |                                |
|-----|--------------------------------|
| INM | interkinetic nuclear migration |
| CNS | central nervous system         |
| VZ  | ventricular zone               |

SVZ                      subventricular zone  
OSVZ                    outer subventricular zone

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# Epigenetic Regulation of Adult Stem Cells

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## SUMMARY

The molecular mechanisms orchestrating stem cell fate receives enormous attention from basic and clinical scientists alike. This attention is caused by the aim to elucidate how stem cells coordinate their unique biological potential to self-renew with their capacity to differentiate into mature tissue cells. Clinicians hope to exploit stem cells and their functional programs for cell replenishment and tissue regeneration in patient cure. Moreover, stem cells can be the origin of cancer, and understanding how stem cells became malignant is essential to develop novel therapies. In recent years, epigenetics took centre stage in stem cell research because there is increasing evidence that stem cells orchestrate their fate-determining gene programs mainly through epigenetic alterations of chromatin. This notion is supported by the discovery of multiple disease-causing mutations in genes encoding epigenetic modifying proteins. In this chapter, we will review the current knowledge on how histone modifications and DNA methylation control

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*List of abbreviations after the text.*

adult stem cells during tissue homeostasis and disease. We will focus primarily on stem cells of the hematopoietic system, as these are the most intensively characterized somatic stem cells, and are still the only ones routinely used in the clinic for transplantation therapy.

## INTRODUCTION

Somatic stem cells possess the capacity to balance their potential to (re-) generate all cell types of a given tissue with self-renewal ability to maintain themselves throughout the life-span of an organism. Switching between these modes requires fundamental alterations in their gene expression programs. Upon differentiation, genes maintaining the self-renewal state must be turned off while instead, genes driving stem cells into differentiation must be turned on. These gene expression changes are tightly coordinated by lineage- and state-specific transcription factors which control transcription directly by binding to defined regulatory gene elements (Orkin, 2000). Transcription factors functionally rely on binding to co-factor proteins, most of which alter the chromatin structure and gene activity through epigenetic modifications.

The term “epigenetics” defines the heritable changes in gene expression that take place during cell division without changes in the DNA sequence (Berger et al., 2009). In mammals, DNA methylation, histone tail modifications and nucleosome positioning are the classical epigenetic mechanisms. However, non-coding RNAs such as microRNAs and long intergenic non-coding (linc) RNAs, emerge as an additional layer of epigenetic gene control (Amaral and Mattick, 2008). All of these different epigenetic pathways are tightly interconnected with each other, and may play fundamental roles in disease development such as cancer (Cedar and Bergman, 2009; Esteller, 2011; Feinberg et al., 2006). In contrast to genetic lesions, at least some epigenetic alterations may be possible to reverse, and thus could be easier targets for therapeutic intervention in disease treatment; a fact that has sparked great interest in epigenetic processes within the medical community.

## EPIGENETIC MECHANISMS

Epigenetic chromatin mechanisms are found in many organisms including both prokaryotes and eukaryotes. In addition to transcriptional gene control, they serve multiple functions in the genome, among which are, chromatin compaction, maintenance of genome stability, suppression of homologous recombination between DNA repeats, genome defence and X chromosome inactivation in females (Jaenisch, 1997; Liang et al., 2002; Monk

et al., 1987; Norris et al., 1994). To fulfil all of these biological functions, the epigenetic mechanisms must be highly flexible, tightly controlled and closely interconnected.

## DNA Methylation

Mammals and other vertebrates show methylated DNA almost exclusively at the C5 position of cytosine (5mC), mostly within CpG dinucleotides. In normal mammalian cells, roughly 70–80% of CG dinucleotides are methylated in the genome (Law and Jacobsen, 2010). Stretches of unmethylated sequences are mostly found at CG dense regions (termed CpG islands), which often coincide with gene promoters (Jones and Takai, 2001). In the pre-implantation embryo, almost the entire genome is stripped of DNA methylation. The genome-wide methylation pattern is re-established by a directed massive *de novo* methylation process that occurs after implantation; the pattern thus established is largely maintained during all following cell divisions (Jaenisch and Bird, 2003). Three general components are needed to orchestrate the genomic methylation pattern: First, the methylation marks must be established (written) and maintained. Secondly, they must be read and translated into functional biological information, and thirdly, they must be removed if necessary.

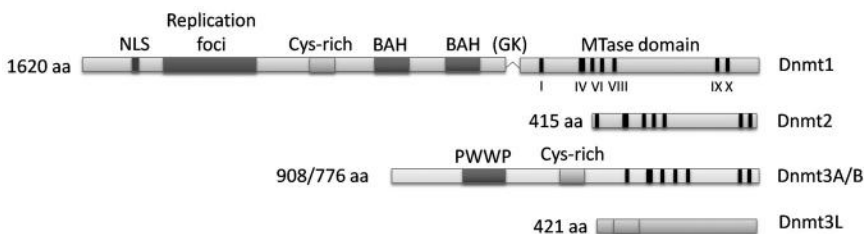
*Writing and maintaining DNA methylation marks* DNA (cytosine-5) methyltransferases (DNMTs) catalyze the covalent addition of a methyl-group to an unmethylated cytosine in an enzymatic reaction that requires S-adenosylmethionine (SAM) as the methyl-group donor (Bestor, 2000; Bird, 2002). In mammals, there are five DNMTs which can be placed into three families based on structural and functional differences.

The first family comprises DNMT3a and DNMT3b and their regulatory factor DNMT3-like protein (DNMT3L). DNMT3a and DNMT3b are believed to be the major *de novo* methyltransferases—they establish the initial addition of methyl-groups onto CpGs in unmethylated DNA double strands (Okano et al., 1999; Okano et al., 1998). DNMT3a and DNMT3b expression patterns parallel the re-establishment of post-implantation DNA methylation during mouse embryogenesis, supporting the notion that they are linked to the early embryonic *de novo* methylation process (Robertson et al., 1999). The third family member, DNMT3L, lacks C-terminal conserved residues required for the methyltransferase activity and also misses the N-terminal PWWP domain, which is believed to be involved in DNA binding (Aapola et al., 2000; Aapola et al., 2001; Qiu et al., 2002). However, in spite of the absence of methyltransferase activity, the phenotype of *Dnmt3L* knockout mice resembles that of the germ-cell-specific conditional knockout of the catalytically active *Dnmt3a* as both mutants have altered imprinted gene-associated *de novo* methylation (Bourc'his et al., 2001; Hata et al., 2002).



DNMT3L co-localizes and co-immunoprecipitates with DNMT3a and DNMT3b and enhances the *de novo* methylation efficiency by stabilizing the conformation of the active centre of both enzymes (Hata et al., 2002; Jia et al., 2007). Intriguingly, DNMT3L was shown to connect *de novo* methylation to histone modification—DNMT3L recognizes the unmethylated histone 3 tail and thus directs the positioning of DNMT3a in the genome for *de novo* methylation (Ooi et al., 2007).

The second family comprises only a single methyltransferase, DNMT1, which is the assigned maintenance enzyme—it copies the methylation pattern of a methylated parent DNA strand to an unmethylated daughter strand after cell division (Yen et al., 1992). DNMT1 was the first mammalian methyltransferase that was purified and cloned (Bestor, 1988). DNMT1 has a 5- to 30-fold preference for hemimethylated DNA substrates as compared to unmethylated DNA *in vitro* (Hermann et al., 2004); its contribution to *de novo* methylation *in vivo* is thought to be minimal, but this has not been firmly established. Homologues of DNMT1 have been found in nearly all eukaryotes whose DNA bears 5mCs, but not in those that lack it. The C-terminus of DNMT1 contains the catalytic methyltransferase domain which is conserved with other methyltransferases including those of prokaryotes (Bestor, 2000). In contrast, the large N-terminal domain is unique and cannot be found in any other methyltransferase. It provides a nuclear import signal, replication-foci-targeting sequences, a zinc-finger domain, a major serine-phosphorylation site and a proliferating-cell-nuclear-antigen-interacting motif (Bestor, 1992; Chuang et al., 1997; Glickman et al., 1997; Leonhardt et al., 1992; Liu et al., 1998). During the S-phase of cell cycle, DNMT1 is recruited to replication foci at which it copies the methylation pattern present on the parental strands onto the daughter strands (Leonhardt et al., 1992).



**Figure 1. Classes of mammalian DNA methyltransferases (DNMTs).** The murine protein structures are depicted; human DNMTs are highly conserved. NLS: nuclear localization sequence; replication foci: replication-foci-targeting sequence; MTase domain: methyltransferase domain; I–X: conserved catalytic motifs; BAH: bromo-adjacent homology domain. Dnmt3L family lacks C-terminal conserved residues required for the methyltransferase activity and also misses the N-terminal PWWP domain, which is believed to be involved in DNA binding (Aapola et al., 2000; Aapola et al., 2001; Qiu et al., 2002). Adopted from (Goll and Bestor, 2005).

The third family also consists of a single member, DNMT2, which however has no DNA methyltransferase activity but, has been described to methylate cytosine 38 in the anticodon loop of tRNA<sup>Asp</sup> (Goll et al., 2006; Okano et al., 1998; Yoder and Bestor, 1998).

*Reading and translating DNA methylation: Methyl-CpG binding proteins*  
DNA methylation is usually associated with transcriptionally silent chromatin, and is thought to inhibit gene transcription by two principle mechanisms: First, methylated CpGs may inhibit the interaction of transcriptional activators with their cognate sequences in regulatory DNA elements. Second, methyl-CpGs become bound by specialized proteins that recognize and recruit silencing cofactors. This mechanism involves the methyl-CpG binding domain (MBD) proteins (MDPs) (Hendrich and Bird, 1998). MDPs comprise a family of six different proteins which include Mbd1-4, Mecp2 and Kaiso (Zbtb33). With the exception of Kaiso and Mbd3, all MDPs have a MBD by which they bind to methyl-CpGs (Klose and Bird, 2006). MDPs can mediate transcriptional silencing by targeting multiprotein complexes containing chromatin remodelling proteins and histone deacetylases (HDACs) to methylated DNA. A salient example is the recruitment of the Mi-2/NuRD complex by MBD2, which includes HDACs 1 and 2 and the Mi-2 protein, a member of the Swi2/Snf2 superfamily of ATPases that disrupt histone-DNA interactions leading to silencing of gene transcription (Wade et al., 1999; Zhang et al., 1999).

*Erasing DNA methylation marks* DNA methylation can be lost either passively during DNA replication in cell division when maintenance methylation is inhibited, or by an active process when 5mC is enzymatically removed. While active demethylation occurs at the genome-wide scale in primordial germ cells and early embryos, it can be detected in a locus-specific manner in somatic cells. In fact, active demethylation has been proposed to control transcriptional activity of selected genes (Bruniquel and Schwartz, 2003). The mechanism of active demethylation in mammals is poorly understood and still a subject of debate (Gehring et al., 2009; Ooi and Bestor, 2008). In contrast, this process is much better understood in plants, in which DNA glycosylases—normally involved in DNA repair—recognize and remove 5mC from the DNA, leading to the replacement with an unmethylated cytosine (Zhu, 2009).

In mammals, three conceptually different mechanisms have been proposed to exert active DNA demethylation: direct removal of the methyl-group from the 5C position of cytosine, base excision repair, and nucleotide excision repair (Bhattacharya et al., 1999; Gehring et al., 2009). Indeed, direct evidence for the involvement of base excision repair has recently been demonstrated in zebra fish in that overexpression of AID (Activation-induced deaminase) and Mbd4 causes demethylation, a process facilitated by Gadd45 (Growth arrest and DNA damage-inducible alpha) (Rai et al.,

2008). Another report showed that active demethylation can also be induced upon hormone treatment in a process that requires the DNA glycosylase activity of Mbd4 (Kim et al., 2009). Very recently, a great focus lies on the role of Ten-eleven translocation (TET) proteins and their oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) as a possible intermediate in DNA demethylation.

*Ten-eleven translocation (TET) proteins and 5-hydroxymethylation* Human TET1 was discovered as a gene that is fused to the mixed lineage leukemia (MLL) gene in leukaemia patients with t(10;11) translocation (Lorsbach et al., 2003). By now, three TET family members are known from mammals: TET1, TET2 and TET3, all of which have a high degree of homology within their C-terminal catalytic domain (Iyer et al., 2009; Loenarz and Schofield, 2009). TET proteins catalyze oxidation of 5mC to 5mC derivatives, including 5-hydroxymethylcytosine (5hmC). The 5hmC mark was initially identified in the T-even bacteriophage more than half a century ago (Wyatt and Cohen, 1953), but only recently it was found to exist also in vertebrate cells (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). A number of recent studies suggested a major biological role of 5hmC and TET proteins in gene regulation, development and disease. TET-mediated conversion of 5mC to 5hmC in ES cells is preferentially located at the bodies of actively transcribed genes (Ficz et al., 2011; Wu et al., 2011a; Wu et al., 2011b). How 5hmC increases transcription is not yet known, but as mentioned above, oxidation of 5mC to 5hmC by TET enzymes may create an intermediate during DNA demethylation, which subsequently may be deaminated to 5-hydroxyuracil (5hmU) by AID and removed by base excision repair (Guo et al., 2011). When the base is excised, it is replaced by unmethylated cytosine promoting gene expression. It is also possible that 5hmC functions as a new epigenetic mark which controls gene function more directly by recruiting specific reader proteins (which have not yet been identified) similar to the reading of 5mC by MDPs (which are not able to bind 5hmC) (Jin et al., 2010). However, even if these 5hmC reader proteins do not exist, conversion of 5mC to 5hmC can obviously repel 5mC readers from the DNA, and as a consequence is likely to reverse many of the cellular effects of cytosine methylation.

Major insight into the biological role of TET proteins came from generation of knockout mice. Tet1 and Tet2 are highly expressed in primordial germ cells, whereas Tet3 is highly expressed in oocytes (Tan and Shi, 2012). However, knockout of Tet1, Tet2 or Tet3 alone revealed no significant effects on both primordial germ cells and oocytes, suggesting a marked redundancy between Tet proteins during early mouse development (Tan and Shi, 2012). In contrast, there appear to be specific effects of Tet proteins during later developmental and postnatal stages. Tet3 knockout mice are neonatal lethal, showing a non-redundant function during organ

development (Gu et al., 2011). In contrast, Tet1 knockout mice are smaller but otherwise develop normally (Dawlaty et al., 2011). Importantly, knocking out Tet2 demonstrated its role in hematopoiesis and cancer development (Ko et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). Tet2 knockout mice have increased self-renewal of hematopoietic stem cells, perturbed myeloid and lymphoid differentiation, and develop myeloid neoplasms. This is interesting because TET2 somatic deletions and loss-of-function mutations have been identified in patients with myelodysplastic syndromes (MDS) and myeloid leukemia (Abdel-Wahab et al., 2009; Delhommeau et al., 2009; Jankowska et al., 2009; Langemeijer et al., 2009). Although much work is still needed to fully understand the molecular and biological functions of TET proteins, it is clear that they play important roles in controlling gene expression during development, tissue homeostasis and disease, most likely by their capacity to convert 5mC to 5hmC.

*Mapping DNA methylation marks* An important prerequisite for understanding the biological role of DNA modifications such as 5mC and 5hmC is to know their locations within the genome. While the genomic sequence is usually stable, the epigenome is highly dynamic and can undergo vast remodelling during cellular development and diversification, thus providing significant challenges for their mapping. It has been known for quite some time that the 5mC marks are unevenly distributed at the genome, and that this normal distribution is non-randomly perturbed in many cancers or other diseases (Sandoval and Esteller, 2012). Similarly, 5hmCs appear to have characteristic localizations (Booth et al., 2012), which are likely to be remodelled in cancer as well. Consequently, the distribution of DNA modification conveys important insight into how epigenetic information controls the biology of cells during development and disease.

Classically, 5mC has been assessed by bisulphite-treatment of DNA (which deaminates unmodified Cs into thymines (Ts) more quickly than modified Cs) followed by analyses with standard molecular biology tools such as PCR, cloning in bacteria and Sanger sequencing. A major improvement of these locus-specific efforts came by the introduction of microarrays allowing global profiling of 5mC marks. However, such arrays do not offer single-base information and are not quantitative. These drawbacks have just recently been overcome by analysing bisulphite-converted DNA with massive parallel sequencing platforms (Laird, 2010). Obviously, while the ideal approach is to map all 5mCs by whole genome sequencing (Lister et al., 2009); this is still too costly and requires high amounts of starting material and significant bioinformatic capacity.

Reduced representation bisulphite sequencing (RRBS) provides an instrumental alternative strategy which allows wide coverage of CpGs

across the genome (Meissner et al., 2008). In RRBS, bisulphite-treated DNA is cut with an endonuclease recognizing CG-dinucleotides, size fractionated on a gel and subsequently deep sequenced from the digested DNA ends. However, RRBS is biased towards CpG rich regions, thus information on methyl changes in other genomic regions is more limited. An improved version of RRBS was recently introduced to provide better coverage of non-island CpGs by generating longer sequence reads and application of a modified bioinformatics pipeline (Akalin et al., 2012). Finally, genomic 5mCs (or 5hmCs) can also be analysed at genome scale by enrichment with specific antibodies or affinity proteins before sequencing or array hybridization (Schmidl et al., 2009; Weber et al., 2005). Importantly, standard bisulfite sequencing cannot distinguish between 5mC and 5hmC. While there are now methods for distinguishing between these marks (Booth et al., 2012; Pastor et al., 2011; Song et al., 2012), they currently require large amounts of material and expense, thus currently limiting our ability to map and understand their distinct biologic roles.

## **Histone Modifications**

The DNA of higher eukaryotes is densely packed in chromatin to fit into the nucleus. However, compaction must be flexible to permit spatiotemporal access to DNA by regulatory proteins. Pioneering studies more than three decades ago used DNase 1 hypersensitivity to introduce the principle that actively transcribed genes contain an “open” chromatin structure (Weintraub and Groudine, 1976). Consequently, DNA compaction must be highly dynamic to allow local switching between “open” and “closed” states. The smallest packaging unit is the nucleosome, which consists of an octamer of core histones of two each of H2A, H2B, H3 and H4, around which 147 bp of DNA are wrapped in superhelical turns.

Over the last couple of decades it became clear that in addition to DNA compaction, the nucleosome is a sophisticated and highly dynamic signalling molecule with an essential function in orchestrating gene expression states (Turner, 2012). These dynamics are caused by nucleosome structural variability and a large number of post-translational modifications of the core histones, which include acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines and threonines, as well as ubiquitination and sumoylation (Kouzarides, 2007). Gene expression states appear mainly influenced by methylation and acetylation at the N-terminal histone tails, which account for approximately a quarter of the total amino acid content of the core histones. Addition or removal of acetyl- or methyl-groups is conducted by specific classes of enzymes: histone acetyltransferases, histone deacetylases, histone methyltransferases and histone demethylases.

*Histone acetylation* It is believed that histone acetylation is generally linked to transcriptional activation (Allfrey et al., 1964; de Ruijter et al., 2003; Ito et al., 2000; Karlic et al., 2010; Kurdistani et al., 2004; Wade, 2001). Histone tails are normally positively charged, which is neutralized by acetylation. As a consequence, acetylation decreases the ability of the histones to bind to DNA and thus decompacts the chromatin to provide easier access for transcriptional activator proteins. Moreover, acetylation-modifying enzymes also interact with the DNA methylation machinery and with other pathways of chromatin modification to further enhance the regulatory alterations (de Ruijter et al., 2003). Finally, lysine acetylation/deacetylation can alter DNA binding, activation, stability, nuclear localization, and coactivator interaction of non-histone transcriptional activators (Sternier and Berger, 2000), all contributing to regulating gene expression.

The acetylation of lysine residues by histone acetyltransferases (HATs) is among the best-characterized histone modifications. HATs use Coenzyme A (CoA) as a donor to transfer an acetyl group to histones as well as to other proteins such as transcription factors. HAT proteins are grouped into at least six different families (Marmorstein, 2001). One family is comprised of the closely related Creb Binding Protein (Cbp, Crebbp) and p300 (Ep300), both of which are mammalian co-activators of transcription that can acetylate all four core histones (Kalkhoven, 2004). A second group of HATs belongs to the Myst family, which includes Tat Interacting Protein 60 (Tip60, Kat5) and MOZ, a regulator of hematopoiesis which was identified as a chromosomal translocation partner in human leukemia (Voss and Thomas, 2009).

Histone deacetylases (HDACs), which are important for gene repression, are grouped into four classes depending on sequence identity and domain organization (de Ruijter et al., 2003; Wade, 2001). Classes I, II, and IV contain the classical HDACs which can be inhibited with trichostatin A (TSA). The class I HDACs HDAC1 and HDAC2 are major components of several well-known multi-protein complexes with potent transcriptional repressor activity such as the NuRD/Mi2 complex (Zhang et al., 1999).

*Histone methylation* Histone methylation can be associated either with active or with repressed chromatin, dependending on the position of the lysine or arginine that is methylated (reviewed in Kouzarides, 2007). Methylation can occur at lysine (K) or arginine (R) residues of histones and other proteins. Lysines can be monomethylated (me1), dimethylated (me2) or trimethylated (me3), and arginines can be dimethylated in a symmetrical or asymmetrical fashion. Trimethylation of H3K4 is often found at actively transcribed or poised gene promoters, while active or poised enhancers are ususally marked by monomethylation, but not trimethylation, of H3K4 (Heintzman et al., 2007). Therefore, variations in histone methylation can be used to map distinct genomic regulatory elements.

Two major types of histone methyltransferases (HMTs) exist: arginine-specific or lysine-specific HMTs (which can be SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain containing or non-SET domain containing) (Kouzarides, 2007). In both types of HMTs, cofactor S-Adenosyl methionine serves as a methyl group donor. Most HMTs have a high specificity for a lysine or arginine at a defined position within a histone tail. For example, the mammalian trithorax homologue Mixed lineage leukemia (MLL), a frequent chromosomal translocation partner in pediatric leukemias (Ziemin-van der Poel et al., 1991), methylates exclusively H3K4 to activate gene transcription (Krivtsov and Armstrong, 2007).

Histone demethylases also perform critical roles. Lysine-specific demethylase 1 (Lsd1) was the first histone demethylase (HDM) to be described (Metzger et al., 2005; Shi et al., 2004). Lsd1 is an amine oxidase that catalyzes lysine demethylation in a flavin adenine dinucleotide (FAD)-dependent manner. It is specific for demethylation of H3K4 and H3K9 mono- and dimethylation marks. A second class of histone demethylases encompasses a large protein family of Jumonji C (JmjC) domain containing proteins, which each has specificity for demethylation of one or two different lysines (Kouzarides, 2007; Metzger and Schule, 2007).

## **Polycomb Genes**

Gene expression programs usually become more restricted during development. Many genes that are expressed in early developmental states are turned off later in a durable and long-lasting way that is transmitted to cellular progeny (i.e., cellular memory). Such heritable changes do not entail mutations or changes in the DNA sequence, but are epigenetic in that they involve changes or modifications in the proteins that organize the chromatin. This epigenetic memory of repressed gene states is mediated by Polycomb genes (PcGs). PcGs were initially discovered in *Drosophila* as repressors of Homeotic genes, which are required for establishing the body axis (Lewis, 1978). They counteract Trithorax proteins (i.e., MLL proteins) which keep activated Homeotic (Hox) genes in an active state.

PcGs are evolutionarily conserved from yeast to mammals. In mammals, they transcriptionally regulate Hox genes to orchestrate formation of the anterior-posterior axis during embryogenesis and to control stem cell function (Gould, 1997; van der Lugt et al., 1994). They form multimeric protein complexes that can organize and modify the structure of chromatin (Sparmann and van Lohuizen, 2006). Two types of complexes have been identified: Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2). PRC2 is considered the initiation complex as it possesses catalytic activity for trimethylation of H3K27 (H3K27me3), a repressive histone mark essential for PcG mediated gene silencing (Simon and

Kingston, 2009). PRC2 has three core components, which in human cells are Enhancer of Zeste (EZH2), Extra sex combs (EED), and Suppressor of Zeste 12 (SUZ12) (Simon and Kingston, 2009). EZH2 contains the catalytic activity for H3K27 trimethylation, which is stimulated by EED and SUZ12 (Konuma et al., 2010).

PRC1 is considered the maintenance complex as it binds to PRC2-induced H3K27me3 to exert gene silencing. PRC1 consists of four core components, which in humans are the Sex Combs Extra factor RING1B, Posterior Sex Comb (BMI1), Human Polyhomeotic (HPH), and Polycomb PC (CBX) (Sparmann and van Lohuizen, 2006). CBX proteins contain a chromodomain to bind H3K27me3. The functional chromatin repressive unit of the PRC1 complex is RING1B. RING1B is an E3 ubiquitin ligase that can monoubiquitinate histone H2A at lysine 119, which has been shown to play a central role in PcG mediated gene repression. It is assumed that H2A ubiquitination can repress transcription by blocking the movement of RNA polymerase (Stock et al., 2007). An alternative explanation of how PcGs silence target genes is through mediation of chromatin compaction (Francis et al., 2004).

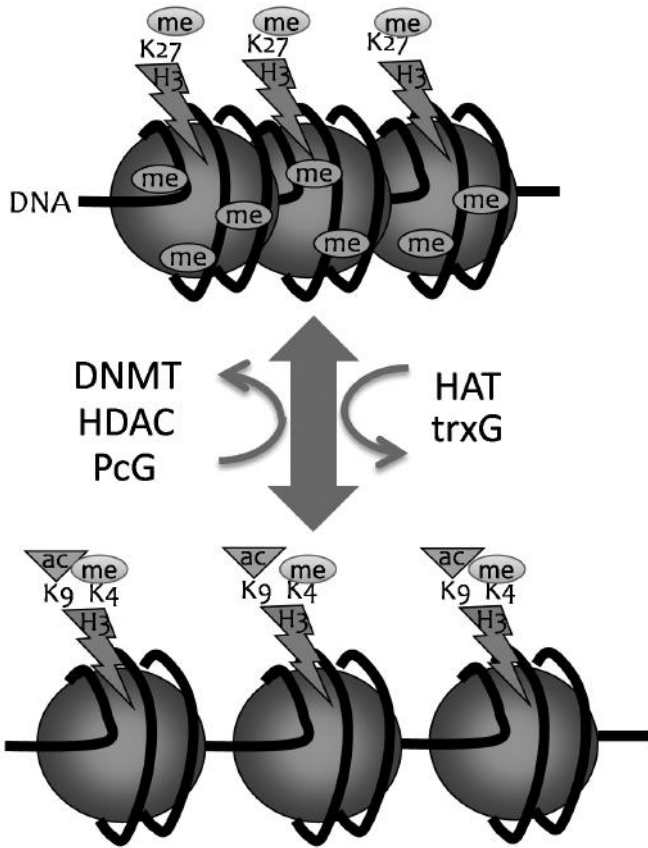
In *Drosophila*, PcG proteins are recruited to chromatin at defined sites termed Polycomb response elements (PREs) (Muller and Kassisi, 2006). However, PREs are still poorly characterized in mammals, and an alternative model of PRC2 binding to chromatin has been suggested. In this model, PRC2 is recruited to defined chromatin positions by interaction with accessory factors harboring sequence-specific DNA-binding capacity, such as the Ying Yang 1 (YY1) protein or transcription factors (Carette et al., 2004; Endoh et al., 2008). Finally, a role of long non-coding RNAs has been suggested to facilitate PRC2 binding (Rinn et al., 2007; Zhao et al., 2008).

## **EPIGENETIC CONTROL OF TISSUE AND CANCER STEM CELLS**

Stem cells are defined by their combined ability for self-renewal and differentiation, usually into multiple types of differentiated progeny (Weissman, 2005). In mammals, somatic ("adult") stem cells have been identified in most organs, and are responsible for tissue regeneration and repair during homeostasis and after injury throughout life. In contrast to embryonic stem (ES) cells, which are derived from the early blastocyst, somatic stem cells are restricted in their developmental potential, such that they can generally regenerate only cells of the tissues in which they reside. Most, but not all somatic stem cells also share a life-style of extended periods of dormancy, followed by activation and proliferation (Trumpp et al., 2010). These periods of dormancy are thought to be important for long-term maintenance of the stem cell population.



## Transcriptionally inactive chromatin



## Transcriptionally active chromatin

**Figure 2. Reversible epigenetic switching between transcriptionally active and inactive chromatin states.** The inactive state is characterized by densely packed nucleosomes (green balls), polycomb-induced H3K27 trimethylation and HDAC-mediated deacetylation, as well as DNMT-induced DNA methylation. In contrast, the transcriptionally active state is characterized by loosely packed nucleosomes, acetylation of H3K9 and trithorax-mediated methylation of H3K4.

*Color image of this figure appears in the color plate section at the end of the book.*

Over the past decade, much excitement has been generated by applying the concepts of general stem cell biology to cancer (Dick, 2008). Essentially, the idea holds that a malignancy is maintained by stem cell-like cells. These so called cancer stem cells (CSCs) share features with normal stem cells in their ability to generate more CSCs, and can also regenerate that cancer

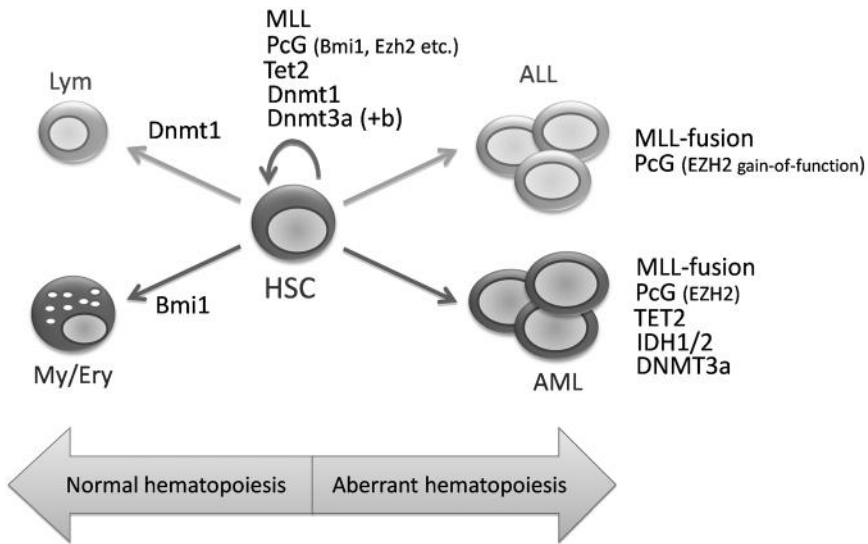
after chemotherapy or after experimental transplantation into another host (Reya et al., 2001). Acute myeloid leukemia (AML), a fatal hematopoietic neoplasm in which malignant cells of the myeloid lineage are blocked in differentiation, was among the first cancer type for which CSCs have been described (Bonnet and Dick, 1997; Lapidot et al., 1994; Magee et al., 2012). Later on, CSCs were identified in many other cancers as well, such as breast cancer (Al-Hajj et al., 2003), colon cancer (Dalerba et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007), brain cancer (Bao et al., 2006; Piccirillo et al., 2006; Singh et al., 2004) and others. When tested experimentally by transplantation, the cells that can re-initiate the tumor *in vivo* are also referred to as tumor initiating cells. In some cases, CSCs may also share physical markers with normal stem cells, suggesting that CSCs may arise by mutation of normal somatic stem cells (Lapidot et al., 1994). Alternatively, the CSCs may arise from more differentiated progenitors, but re-acquire stem-like features (Cozzio et al., 2003; Huntly et al., 2004).

The existence of CSCs and the genes controlling them are of great clinical relevance, as their unique 'stemness' functions, such as periods of dormancy, probably enable escape from conventional anticancer therapies that are designed to target the rapidly cycling and highly proliferating cancer blasts. This inability to eradicate CSCs might be responsible for the disease relapses that are frequently observed in patients with cancer.

It is worth noting that many of the details of the CSC concept are controversial, leading to heated debates concerning the frequency of the CSCs within a tumor, their markers, and the clinical significance (Kelly et al., 2007; Magee et al., 2012; Quintana et al., 2008). Nevertheless, CSCs are an instrumental concept facilitating the understanding of functional heterogeneities and hierarchies within tumors, and as such are helpful to concentrate clinical and mechanistic efforts on the most relevant tumor cell fractions.

## Epigenetic Control of Hematopoietic Stem Cells

HSCs are the most intensively studied somatic stem cell type in mammals, and are the only stem cells routinely used in the clinic. HSCs can generate almost all hematopoietic lineages, except for some specialized macrophage populations such as microglia (Ginhoux et al., 2010; Orkin, 2000; Schulz et al., 2012). The key challenge for HSCs is to balance their self-renew ability with the need to constantly replenish the high demand of generating new blood cells. This replenishment is carried out by a subpopulation of activated HSCs, whereas most HSCs stay dormant and cycle only very rarely throughout life (Wilson et al., 2008). Thus, longevity and dormancy are essential stem cell hallmarks that compete with activation and production of differentiated daughter cells. Considering the many fate options of a



**Figure 3. Functions in hematopoietic stem cells and mutations in leukemia of epigenetic regulators.** HSCs can differentiate into lymphoid (Lym) or myeloid/erythroid (My/Er) progeny, or after malignant transformation can form acute myeloid (AML) or lymphoid (ALL) leukemias. Indicated is a selection of epigenetic regulators that have been found mutated in human leukemia and/or have experimentally been shown to regulate HSC function in the mouse.

single HSC, the most central question is how stem cells decide which fate option to follow. In recent years, epigenetic mechanisms have entered a central role in orchestrating these stem cell fate decisions during normal hematopoiesis and leukemia.

*DNA methylation in hematopoietic stem cells* Recent research has revealed that DNA methylation is a critical epigenetic mechanism for maintaining the function of HSCs. The first evidence for the function of *de novo* methylation in HSCs came from Nakauchi and colleagues, who observed that compound deletion of both Dnmt3a and Dnmt3b severely inhibited the repopulation function of HSCs (Tadokoro et al., 2007). In that same study, individual deletion of either Dnmt3a or 3b had a minimal effect, suggesting that both enzymes function redundantly. In contrast, Goodell and colleagues found that when Dnmt3a was deleted and the HSCs were transplanted serially into multiple rounds of recipients, the HSCs ultimately lost their ability to differentiate, but retained remarkable self-renewal capacity, expanding dramatically (Challen et al., 2012). Analysis of DNA methylation and gene expression changes in the mutant HSCs and their progeny suggested that in the absence of Dnmt3a, the stem cell-specific genes were unable to be repressed, allowing the self-renewal state of the HSCs to be sustained and inhibiting differentiation. Strikingly, mutations in DNMT3a are found in

nearly 20% of AML patients (Ley et al., 2010). The mutations in these patients are typically heterozygous missense mutations in the catalytic domain. Whether these mutations are functionally similar to the null mutation in the Dnmt3a KO HSCs is unclear. However, it is likely that the AML mutations somehow lead to ineffective differentiation which, in cooperation with additional mutations, promotes malignant transformation.

A more general role for DNA methylation in HSCs was revealed using Dnmt1 mutant mice. Conditional ablation of Dnmt1 in HSCs leads to severe genomic demethylation, which is followed by massive apoptosis-induced bone marrow pancytopenia and a rapid death of the animals (Broske et al., 2009). A similar importance of Dnmt1 in hematopoietic cells has been shown in a second study (Trowbridge et al., 2009). An instrumental tool for deciphering the role of DNA methylation in tissues stem cells are mice with hypomorphic Dnmt1 expression (Gaudet et al., 2003). In these animals, Dnmt1 is gradually knocked down by combining a hypomorphic Dnmt1<sup>chip</sup> (chip=cDNA homologous insertion protocol) allele with a knockout (c) allele (Tucker et al., 1996). Dnmt1<sup>c/chip</sup> mice express approximately 10% of Dnmt1 levels found in wild type mice, thus resulting in global genomic hypomethylation. HSCs from the Dnmt1<sup>c/chip</sup> mice have reduced self-renewal capacity, and fail to suppress key myeloerythroid regulators and as a consequence can differentiate into lineage-specified myeloerythroid progenitors but are blocked in their differentiation into lymphoid progeny (Broske et al., 2009; Vockentanz et al., 2010). Congruently, Dnmt1-controlled methylation has a key role in lineage differentiation decisions of stem cells. Importantly, this notion has recently been confirmed and extended by showing that the genome of myeloid cells is less methylated than that of lymphocytes, and that treatment of HSCs with a demethylating agent induces myeloid over lymphoid differentiation (Ji et al., 2010). Moreover, high-resolution methylome maps using shotgun bisulfite sequencing further confirmed the major role for DNA methylation in myeloid/lymphoid lineage specification of hematopoietic stem and progenitor cells (Hodges et al., 2011).

Taken together, functional studies in mice together with genome-scale mapping of methyl-CpG marks have revealed a specific role for DNA methylation in the control of HSC fate. From these data, a model can be derived in which DNA methylation is an essential epigenetic mechanism to dynamically drive self-renewal and lineage fate decisions by silencing fate-opposing gene programs. Thus, DNA methylation is likely to act as gate-keeper of HSC multipotency. This notion is further supported by experiments using mice in which the hypomorphic Dnmt1 allele has been combined with a CRE-responsive conditional Dnmt1 knockout allele, showing that HSCs are much more sensitive to DNA methylation defects than committed progenitors (Broske et al., 2009). Thus, it is tempting to

speculate that DNA methylation might have been essential for the evolution of an adaptive immune system from a more primitive innate system by allowing the emergence of a multipotent stem cell state from a more unilateral earlier precursor type.

*DNA methylation in leukemia stem cells* Aberrant DNA methylation patterns are a hallmark of cancer and demethylating agents are being used to treat certain kinds of cancer, such as myelodysplastic syndrome and AML (Bender et al., 1998; Claus and Lubbert, 2003). AML is a genetically heterogeneous disease, which can be driven by a number of different oncogenes. Common to all AMLs is their blocked myeloid differentiation, a large contributor to which is inhibition of key myeloid transcription factors such as C/EBP $\alpha$  and/or PU.1 (Tenen, 2003). Recently, a large cohort of AML patient samples was characterized for their genome-scale methylation patterns, allowing their separation into 16 biological distinct subgroups (Figueroa et al., 2010b). Moreover, that study showed that frequent leukemia-driving oncogenes such as AML1-ETO and PML-RARA are associated with specific methylation profiles. Thus, aberrant DNA methylation appears to play a major part in AML, a notion that is further confirmed by the recent identification of AML-associated mutations in factors regulating the methylome, such as DNMT3A and TET2 (Delhommeau et al., 2009; Langemeijer et al., 2009; Ley et al., 2010).

However, such studies on human patient samples do not establish whether methylation alterations are a cause or a result of the leukemia. Likewise, the contribution of the aberrant methylation patterns to the leukemia stem cells (LSC) was not addressed. These questions were experimentally investigated in mice by inducing AML in *Dnmt1* mutant stem and progenitor cells through retroviral expression of MLL-AF9, the fusion product of the t(9;11)(p22;q23) translocation (Broske et al., 2009; Trowbridge et al., 2012). This model revealed a profoundly reduced capability of transformed hypomethylated cells to acquire and maintain malignant self-renewal properties, indicating that, similar to normal stem cells, LSC potential depends on constitutive methylation levels. A myc-driven leukemia model supported this notion. Ectopic co-expression of myc and bcl2 in wild-type stem cells induces a mixed B lymphoid/myeloid leukemia (Broske et al., 2009). In contrast, myc/bcl2 transformed DNA hypomethylated stem cells specifically lost the capacity to develop B lymphoid leukemia, while myeloid leukemia was retained. Consequently, the capacity of LSCs to exert multilineage fate programs requires constitutive methylation. Based on these findings it can be concluded that both normal and malignant stem cells share methylation-dependent control mechanisms to exercise common functional properties. However, the molecular basis and methylation controlled target genes may, at least in part, differ between HSCs and LSCs. This notion is supported by a recent

study from Orkin and colleagues, who suggested that MLL-AF9+ LSCs depend on DNA methylation-mediated silencing of a specific gene signature that is marked by bivalent chromatin domains (Trowbridge et al., 2012). Consequently, it may be possible to explicitly target stem cell potential in leukemia by a combined therapeutic strategy involving demethylating agents together with drugs that selectively target individual methylation controlled genes.

*5-Hydroxymethylation, hematopoietic stem cells and leukemia*  
5-Hydroxymethylation is catalyzed by the TET oncogene family (Tahiliani et al., 2009). Recently, the TET2 gene was identified to be mutated in a variety of myeloid disorders, with 7% to 23% in *de novo* AML and 14% to 55% in other myeloid malignancies (Delhommeau et al., 2009; Langemeijer et al., 2009). Interestingly, Melnick and colleagues reported that activating mutations of isocitrate dehydrogenases IDH1 and IDH2 are mutually exclusive with mutations of TET2 (Figueroa et al., 2010a). The catalytic function of TET proteins is dependent on 2OG ( $\alpha$ -ketoglutarate) which is produced by IDH enzymes (Ward et al., 2010). IDH mutations, however, lead to the aberrant production of 2-hydroxyglutarate (2HG), which may interfere with the 5hmC catalytic activity of TET2. In line with this notion is the observation that IDH mutations are associated with similar epigenetic defects as TET2 mutations; thus, mutation of TET2 and IDH1/2 appear to be genetically redundant (Figueroa et al., 2010a).

The causal role for TET mutations in myeloid transformation and stem cell function has recently been confirmed by two independently generated conditional Tet2 knockout mice (Moran-Crusio et al., 2011; Quivoron et al., 2011). These animals showed that Tet2 loss in the hematopoietic compartment leads to increased stem cell self-renewal and a progressive enlargement of the hematopoietic stem/progenitor cell compartment. Eventually, the mice went on to develop a myeloproliferative phenotype. Interestingly, even heterozygous Tet2 loss caused stem cell expansion. Thus (monoallelic) TET2 mutations in humans may contribute to hematopoietic transformation by increasing the HSC pool size, thereby putting the HSCs at higher risk to acquire secondary mutations which transform them into frank LSCs.

*Polycomb genes in hematopoietic and leukemic stem cells*  
Maintenance of an epigenetic stem cell memory is mediated by PcGs (Valk-Lingbeek et al., 2004). In the case of HSCs, the first evidence for a functional role of PcGs came from mice deficient for the Polycomb repressive complex 1 (PRC1) component Bmi1 (Simon and Kingston, 2009). Bmi1 is a co-factor for the E3 ubiquitin ligase activity of RINGA/B and involved in nucleosome compaction (Konuma et al., 2010; Valk-Lingbeek et al., 2004). It is highly expressed in purified HSCs, and although Bmi1 deletion has no major effect on fetal hematopoiesis, the bone marrow of adult mice becomes progressively pancytopenic (Iwama et al., 2004; Oguro et al., 2006; van der

Lugt et al., 1994). Detailed analyses have shown that *Bmi1*<sup>-/-</sup> HSCs have impaired long-term self-renewal capacity (Iwama et al., 2004; Lessard and Sauvageau, 2003; Park et al., 2003). Reciprocally, enforced expression of *Bmi1* leads to a marked expansion of HSCs by enhancing self-renewal, indicating that the level of self-renewal is directly dependent on the expression level of *Bmi1* (Iwama et al., 2004). How does *Bmi1* control HSCs? Interestingly, although Hox genes are the major targets of PcGs during early embryogenesis, in HSCs this appears not to be the case. Hox expression is not significantly dysregulated in HSCs lacking *Bmi1* or other PcG genes such as *Mph1/Rae28* or *Eed* (Lessard and Sauvageau, 2003; Park et al., 2003). Instead, *Bmi1* controls cell cycle genes in HSCs, in particular the *Ink4a/Arf* (*Cdkn2a*) locus (Iwama et al., 2004; Jacobs et al., 1999; Park et al., 2003). The *Ink4a/Arf* locus encodes p16<sup>Ink4a</sup>, a cell cycle-dependent kinase inhibitor, and the tumor suppressor p19<sup>ARF</sup> in the mouse or p14<sup>ARF</sup> in humans, respectively. Enhanced expression of *Ink4a/Arf* in *Bmi1*<sup>-/-</sup> mice results in a cell cycle arrest and p53 dependent induction of apoptosis. The *Ink4a/Arf* locus seems to be the major target of *Bmi1* in controlling HSCs, as the *Bmi1*<sup>-/-</sup> hematopoietic phenotype could at least in part be rescued by deleting *Ink4/Arf* (Oguro et al., 2006). In addition to its role in self-renewal regulation of HSCs, *Bmi1* also controls multi-lineage differentiation potential. *Bmi1*<sup>-/-</sup> HSCs undergo accelerated B lymphoid specification by a mechanism that involves enhanced expression of the B cell transcription factors *Ebf1* and *Pax5* (Oguro et al., 2010). This is induced by loss of the repressive H3K27me3 mark on regulatory elements of these genes, leading to their premature activation in stem cells.

Deletion of other PcGs cause effects in the hematopoietic system as well. For example, fetal liver HSCs deficient for the PRC1 protein *Mph1/Rae28* decreased over time to less than 20-fold of the number found in wild type controls (Kim et al., 2004; Ohta et al., 2002). Interestingly, heterozygous deletion of the PRC2 gene *Eed* causes a severe myelo-lymphoproliferative disease in mice, suggesting a role for PcGs in hematopoietic neoplasms (Lessard et al., 1999). However, some PcG-deletions, such as *Mel18* or *Cbx2/M33*, do not show any gross abnormalities in the hematopoietic system, indicating that there is also redundancy among individual PcG group members (Iwama et al., 2004; Konuma et al., 2010).

Interestingly, a few years ago Laird and colleagues discovered that genomic PcG target regions identified in human ES cells are more likely to have cancer-specific promoter DNA hypermethylation than non-target regions, suggesting a link between PcG genes and the stem cell-related epigenetic landscape of cancer cells (Widschwendter et al., 2007). The role of PcG genes in controlling malignant stem cell properties is best understood by the example of *Bmi1* in mice. CSCs, similar to normal stem cells, share a high dependency on *Bmi1* to maintain their self-renewal capacity. In

a mouse model of AML, the proliferation of LSCs is promoted by Bmi1 (Lessard and Sauvageau, 2003). In humans, high expression of BMI1 is associated with an unfavorable prognosis of AML patients (Mihara et al., 2006). Furthermore, knockdown of BMI1 by RNA interference impairs self-renewal of both normal human cord blood CD34+ cells as well as primary AML CD34+ cells from patients (Rizo et al., 2009). Thus, Bmi1 appears to play an important role not only in mouse leukemia but also seem to contribute to human LSC function.

*Histone modifications and trithorax genes in hematopoietic stem cells* ES cells can contain bivalent chromatin structures, which mean that activating H3K4me3 and repressive H3K27me3 histone marks co-exist on the same locus (Bernstein et al., 2006). Subsequently, bivalency has also been identified at the chromatin of selected genes in somatic stem cells such as HSCs (Cui et al., 2009; Weishaupt et al., 2010). The bivalent structure is thought to poise genes involved in differentiation for rapid activation (by losing the repressive H3K27me3 mark) or permanent silencing (by losing the activating H3K4me3 mark). This view is supported by the finding that bivalent chromatin marks are resolved into monovalent marks upon stem cell differentiation (Bernstein et al., 2006). While the repressive marks of these bivalent structures are placed on the chromatin by PcGs, the activating marks are placed and maintained by Trithorax genes (trxGs).

TrxGs were identified in *Drosophila* as antagonists of PcGs, as they activate homebox genes during segmentation in a manner that is inheritable through multiple daughter cell divisions (Gould, 1997; Mozer and Dawid, 1989). The 5 members of the Mixed lineage leukemia (MLL) family, that is MLL1-5, are mammalian trxGs sharing a homologous SET domain (Liu et al., 2009). MLL1-4 are histone methyltransferases specific to methylate H3K4. Because mice deficient for MLL1 are embryonically lethal (Yu et al., 1995), conditional knockout animals have been particularly instrumental to uncover the important role of MLL1 in adult hematopoiesis (Jude et al., 2007). MLL1 is essential to maintain adult HSCs and early progenitors, and ablation of MLL1 results in a rapidly fatal bone marrow hypoplasia. Transplantation experiments revealed that MLL1 is important to preserve HSC self-renewal activity and cell cycle quiescence. The mechanism by which MLL1 controls HSCs appears to involve, at least in part, Hox gene activation. Hox genes, similar to MLL1, are important regulators of HSC self-renewal maintenance (Abramovich and Humphries, 2005). In line with the findings on MLL1 are those on MLL5. Three independent studies have demonstrated an important role of MLL5 in HSCs (Heuser et al., 2009; Madan et al., 2009; Zhang et al., 2009). In *MLL5*<sup>-/-</sup> mice, long term-HSCs are decreased in number, and are functionally impaired when competing with wild type stem cells in transplantation assays.



Analogous to normal stem cells, establishing and maintaining proper histone modification patterns are also essential for LSCs. Much of what we know about the role of histone modifications on LSCs came from studying MLL1-initiated leukemias. Human MLL1 is located on chromosome band 11q23 which is often involved in translocation in infant leukemias of both the myeloid and lymphoid lineages (Bernt and Armstrong, 2011b). MLL1 has more than 60 known translocation partners in leukemia. Gain-of-function experiments using such leukemia-associated MLL-fusion products have been particularly useful to understand epigenetic gene control mechanisms in LSCs. Retroviral delivery of MLL1-fusion products into mouse HSCs followed by their transplantation cause rapid leukemia development in recipient animals (Cozzio et al., 2003). Intriguingly, several groups have shown that at least some MLL1-fusion products can also transform committed progenitor stages, such as the granulocyte-macrophage-progenitor, by conferring ectopic self-renewal properties to these cells (Cozzio et al., 2003; Krivtsov et al., 2006). However, the capacity to convert non-self-renewing progenitors into self-renewing LSCs appears to require extremely high expression levels of the MLL1-fusion protein as achieved by retroviral vectors. In contrast, knock-in mice expressing MLL-AF9 from the endogenous MLL1 promoter sequences fail to initiate leukemia from committed progenitors (Chen et al., 2008). Moreover, different fusion products of MLL1 were shown to lead to different LSC frequencies, a finding which is supported by the differential disease prognosis of human patients with different MLL1 fusions (Somervaille et al., 2009).

A mechanistic hallmark of how MLL1-fusion products initiate leukemia is by their capacity to activate and maintain expression of Hox genes. Hox genes are molecular targets of MLL1 in normal HSCs as well, but their epigenetic mechanism of activation by MLL1 is very different from that by MLL1-fusion products. During translocation to one of the many fusion partners, MLL1 loses its SET domain, and therefore no longer harbours endogenous HMT activity. Instead, MLL1-fusions recruit unique protein complexes which upregulate transcriptional activity of MLL targets (Bernt and Armstrong, 2011b). One of these protein complexes contains DOT1L, a HMT that modifies H3K79 (Okada et al., 2005). Methylation of H3K79 is linked to transcriptional activation; aberrant recruitment of DOT1L might therefore drive MLL fusion-associated inappropriate gene expression.

## **Epigenetic Control of other Somatic Stem Cell Types**

*Neuronal stem cells* Neural stem cells (NSCs) give rise to the major cell types in the central nervous system (CNS), including neurons, astrocytes, and oligodendrocytes. Although in comparison to HSCs much less is known on how NSCs are epigenetically controlled, great progress has been made

during the last years. A genome-wide study has identified genetic loci carrying both repressive and activating histone marks in NSCs, suggesting that bivalent chromatin is not unique to ES cells and HSCs, but occurs more widely in stem cells (Mikkelsen et al., 2007). However, NSCs carry their bivalent chromatin marks at different gene sets than ES cells. In contrast, ES cell specific genes are occupied only by repressive marks to permanently silence expression of these genes in NSCs (Mikkelsen et al., 2007). Instead, genes required later for neuronal and glial development are marked by bivalent chromatin structures, enabling the rapid expression or silencing during differentiation. In line with these results, the PRC1 component Bmi1 is expressed in neuronal stem and progenitors but not in differentiated neurons. *Bmi1*<sup>-/-</sup> mice exhibit neurological defects, indicating that Bmi1 regulates NSC self-renewal and growth, probably by repressing the expression of the *Ink4a/Arf* locus (Molofsky et al., 2005; Molofsky et al., 2003).

These data show that similar to ES cells and HSCs, PcG/trxG controlled histone modifications are important to control identity, proliferation and differentiation capacity of NSCs. Of similar importance is DNA methylation. During embryogenesis, NSC differentiation into astrocytes is controlled by the gp130-STAT3 signalling pathway (Fukuda et al., 2007; Takizawa et al., 2001). Interestingly, STAT binding sites in the promoter of a STAT3 target, Glial fibrillary acidic protein (GFAP), are methylated in neuroepithelial cells of early mouse embryos (E11.5), but are hypomethylated in later (E14.5) embryos (Namihira et al., 2004; Shimozaki et al., 2005; Takizawa et al., 2001). GFAP is proposed to play a role in astrocyte-neuron interactions, and as such may be important in CNS development. Thus, STAT3 activation of GFAP expression and, as a consequence, astrocyte differentiation, is controlled by stage-dependent timing of methylation.

DNA methylation is also important in the control of adult neurogenesis. This has first been observed by analysis of mice deficient for the methyl-CpG binding domain protein Mbd1. *Mbd1*<sup>-/-</sup> mice do not have detectable defects during early development, but adult *Mbd1*<sup>-/-</sup> NSCs show reduced neuronal differentiation and increased genomic instability (Zhao et al., 2003). A possible mechanism for these defects may be the dysregulation of fibroblast growth factor-2 (Egf2), which is hypomethylated and more strongly expressed in *Mbd1*<sup>-/-</sup> NSCs (Li and Zhao, 2008).

A more direct effect of DNA methylation on adult NSCs was seen from analysis of Dnmt deficient mice. Deletion of Dnmt1 in neuronal progenitors causes hypomethylation in postmitotic neurons in the CNS. As a consequence, these progenitors undergo precocious astroglial differentiation and produce hypomethylated neurons with multiple defects (Fan et al., 2005; Golshani et al., 2005; Hutnick et al., 2009). In line, studies using Dnmt inhibitors in *in vitro* brain slice cultures have identified that DNA methylation could target

genes involved in synaptic plasticity, learning and memory (Levenson et al., 2006; Miller and Sweatt, 2007; Nelson et al., 2008).

DNMT3a is also important for neuronal development. DNMT3a is expressed in both developing and mature CNS. Conditional deletion of *Dnmt3a* in the CNS using specific Cre mouse strains showed a loss of motor neurons and defects in the neuromuscular endplate structure (Nguyen et al., 2007). Finally, mice deficient for both *Dnmt1* and *Dnmt3a* revealed that both proteins have overlapping functions in maintaining DNA methylation and modulating of neuronal gene expression in adult CNS neurons (Feng et al., 2010).

*Epithelial stem cells* Epithelial stem cells are present in mammalian skin, intestine, mammary gland and many other organs (Gu et al., 2010). They replenish epithelial cells that cover the surface and cavities of organs to provide protection, sensation, and absorption capability. Despite the epithelium being one of the major tissue targets of tumorigenesis, studies on epigenetic regulation of epithelial stem cells is scarce. This is caused by the difficulties in isolating these cells and thus obtaining sufficient numbers to perform genome-wide epigenetic profiling studies. One of the early epigenetic studies used electron microscopy to suggest that epithelial chromatin undergoes organizational changes at different stages of differentiation (Chepko and Smith, 1997). Later on, global histone modifications were more systematically examined in mammalian skin using immunostainings (Frye et al., 2007).

Similar to their role in other stem cell types, PcGs were shown to control epithelial stem cells. BMI1 is a downstream target of sonic hedgehog signalling to control human mammary stem cells (Liu et al., 2006). Moreover, *Bmi1* ablation in mice lead to reduced activity of mammary stem cells in transplantation assays by a downstream pathway that includes the *Ink4a/Arf* locus (Pietersen et al., 2008). Expression of another PcG member, *Ezh2*, has been found to mark embryonic progenitor cells of the epidermis. *Ezh2* ablation in mice leads to reduced epidermal progenitor cell proliferation accompanied by higher expression of the cell cycle inhibitors *Ink4a* and *Ink4b* (Ezhkova et al., 2009).

Interestingly, *Ezh2* can recruit DNMTs to its target genes, thus linking H3K27 methylation to DNA methylation (Vire et al., 2006). *Dnmt1* expression is high in epidermal progenitor cells, where it is needed to maintain proliferative capacity and prevent premature differentiation (Sen et al., 2010). Moreover, UHRF1, a component of the DNA methylation machinery that targets DNMT1 to hemimethylated DNA is also necessary to suppress premature differentiation and sustain proliferation of epidermal progenitors. In contrast, *GADD45A* and *B*, which promote active DNA demethylation, are required for full epidermal differentiation gene induction (Sen et al., 2010).

*Muscle stem cells* In response to environmental stress, quiescent muscle stem cells, or satellite cells, are activated and proliferate. At this stage, they can either differentiate and fuse to form muscle fibres, or can self-renew and maintain the muscle stem cell reservoir. Transcriptional control of this process is mainly orchestrated by transcription factors Pax3/Pax7 and MyoD (Perdiguero et al., 2009). The *MyoD* gene is epigenetically controlled by DNA methylation. Early experiments using DNMT1 inhibitors have suggested that demethylation of the *MyoD* promoter can lead to derepression of the *MyoD* gene in fibroblasts, which in turn can drive its transdifferentiation into myoblasts (Lassar et al., 1989; Palacios and Puri, 2006; Taylor and Jones, 1979). However, the physiological role of DNA methylation in muscle cell development is still poorly defined and much work lies ahead.

Better understood is the role of histone modifications. Muscle differentiation-specific gene promoters are marked by repressive histone modifications in muscle stem cells. These genes carry hypoacetylated chromatin and are characterized by PcG-mediated H3K27me<sub>3</sub>. Indeed, as in other stem cell types, chromatin repression by PcGs is thought to be a central mechanism for preservation of muscle stem cell identity and silencing of pro-differentiation genes (Sartorelli and Caretti, 2005). EZH2 is recruited to inactive muscle gene promoters by the transcriptional regulator YY1 which promotes transcriptional repression through H3K27 trimethylation (Caretti et al., 2004). However, further studies are clearly needed to demonstrate whether PRC2-mediated repression is a general mechanism for repression of muscle cell differentiation genes, or whether different methylation promoting complexes regulate different sets of genes (Perdiguero et al., 2009)(Sartorelli and Caretti, 2005).

*Mesenchymal stem cells* Mesenchymal stem cells (MSCs) are a diverse group of stem cells which are receiving a lot of attention due to their potential usefulness in regenerative medicine. Although they are usually quiescent, after tissue injury they proliferate and give rise to lineage-committed progenitors and eventually, terminally differentiated cells. Among the most intensely characterized MSCs are bone marrow MSCs and adipose tissue MSCs (adipose stem cells, ASCs), which differentiate into mesodermal lineages such as adipogenic, osteogenic, and chondrogenic cell types. Information on epigenetic regulation of MSCs is scarce. Using methyl-DNA immunoprecipitation and promoter array hybridization (MeDIP-chip), it was recently reported that bone marrow MSCs, adipose tissue MSCs, and skeletal muscle MSCs share a core set of DNA hypermethylated promoters, suggesting that MSCs from different lineages are epigenetically related and arise from a common precursor (Sorensen et al., 2010a). Moreover, there is evidence for a transcriptionally permissive epigenetic ground state of MSCs, which may be responsible for lineage-priming (Sorensen et al., 2010b).

Histone modifications play a role in MSCs as well. In undifferentiated ASCs, inactive promoters of adipogenic and myogenic genes are enriched in a repressive combination of H3K4m3 and H3K27m3, and the absence of the transcriptionally permissive H3K9m3 mark. H3K4m3 and H3K27m3 co-occupy a fraction of these promoters, but not all of them (Delbarre et al., 2010). In contrast, in keratinocytes, the same adipogenic and myogenic promoters are enriched in trimethylated H3K4, K27, and K9, thus suggesting two distinct epigenetic states of inactive promoters which may be related to the potential for their differentiation (Delbarre et al., 2010). Interesting in this regard is that there is no evidence of EZH2 or BMI1 occupancy at these promoters, suggesting that H3K27me3 can be maintained without EZH2, at least in cultured ASCs.

## CONCLUSIONS

The ability to specifically ablate epigenetic regulators in various adult stem cell populations in mice has generated many insights into the roles of a number of these regulators. Coupled with the recent leap in our ability to profile epigenetic modifications, including histone marks and DNA methylation, genome-wide using high-throughput sequencing, we are beginning to accrue a detailed view of the mechanisms through which these epigenetic regulators exert their effects. Some general themes begin to emerge. First, appropriate epigenetic regulation, including DNA methylation and histone modification, are critical for proper differentiation of embryonic and adult stem cells. However, the specific genes through which these effects are mediated, are generally not clear. Second, aberrations in this level of regulation can lead to malignancies as has been abundantly documented now in hematologic malignancies by the number of mutations in epigenetic regulators that lead to different types of leukemias. Again, the precise mechanisms through which the aberrant proteins lead to their effects are unclear, and still needs much attention. Clearly, a better understanding may lead to new approaches to drug development as was impressively shown in the case of Dot1L inhibitors (Bernt and Armstrong, 2011a; Daigle et al., 2011).

## ABBREVIATIONS

|       |                                              |
|-------|----------------------------------------------|
| DNMT  | DNA methyltransferases                       |
| MBD/P | methyl-CpG binding domain (D)/proteins (P)   |
| HDAC  | histone deacetylases                         |
| RRBS  | reduced representation bisulphite sequencing |
| HAT   | histone acetyltransferases                   |

|      |                            |
|------|----------------------------|
| HMT  | histone methyltransferases |
| MLL  | mixed lineage leukemia     |
| AML  | acute myeloid leukemia     |
| HDM  | histone demethylase        |
| PcGs | polycomb genes             |
| LSC  | leukemia stem cells        |

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# Hematopoietic Stem Cell Aging and Oxidative Stress

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## INTRODUCTION

In humans, hematopoietic tissues generate  $\sim 10^{11}$ – $10^{12}$  mature blood cells per day to maintain normal hematologic functions (Suda et al., 2011). These mature blood cells originate from hematopoietic stem cells (HSCs), which exhibit the essential features of stem cells: self-renewal and differentiation. Extensive previous work reveals that HSCs are not a homogenous cell population, but rather can be classified into at least two HSC populations according to the length of the cell cycle (Wilson et al., 2008). While the majority of HSCs cycle relatively actively, with a dividing interval of 9–36 days, a minority of HSCs are more quiescent, with a longer cell-cycle duration of 56–145 days (i.e., dormant HSCs) (Wilson et al., 2008). Over the two year lifespan of a laboratory mouse, the rapid-cycling HSCs divide  $\sim 20$  times, whereas the slow-cycling HSCs divide fewer than five times. Long-term repopulation of HSCs after bone marrow (BM) transplantation is accomplished by dormant HSCs.

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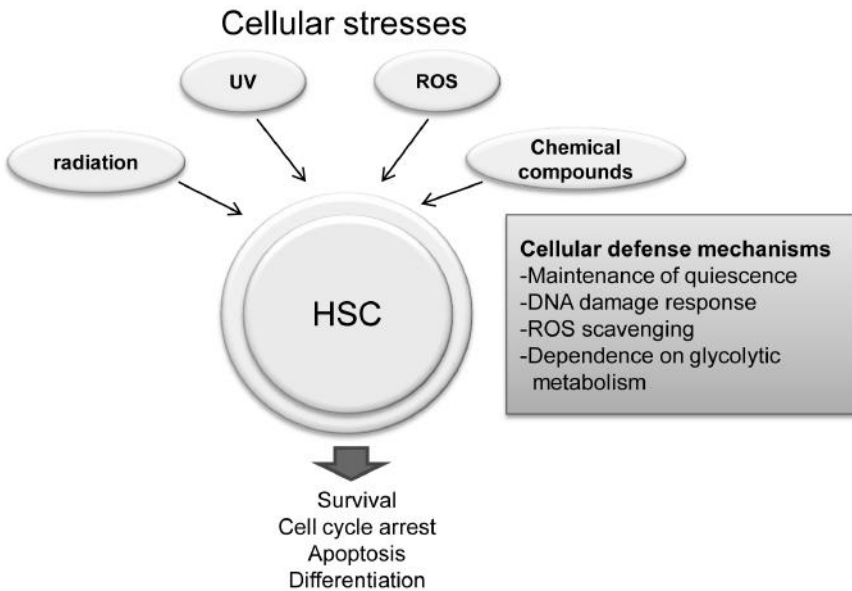
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*List of abbreviations after the text.*

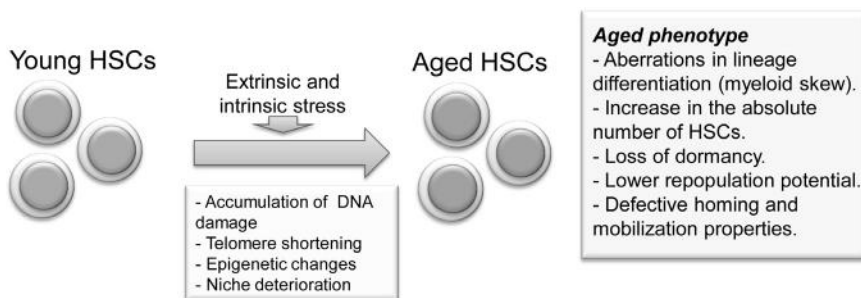
Aging is considered to be the process whereby a living system gradually loses its capacity to maintain homeostasis in response to various stresses (Rossi et al., 2007b). Aging involves accumulation of HSC responses and adaptations to various cellular insults (Figure 1). In principle, HSCs possess limitless self-renewal potential but, in reality, this potential may be limited. Over the course of their lifespan, HSCs are exposed to various stresses, to which they respond using their intrinsic machinery. Meanwhile, the microenvironment surrounding the HSCs may also be altered upon stress, resulting in functional changes that in turn influence HSCs. HSC cell fate is therefore determined by both cell-intrinsic and -extrinsic factors. In this review, we focus on the physiology of aging in the hematopoietic system, and on HSC responses to various cellular stresses such as oxidative stress and DNA damage. A deeper understanding of HSC biology under physiological stress will provide keys to unraveling the pathogenesis of aging, and to treating age-related hematopoietic disorders, such as myelodysplasia syndrome (MDS) and myeloproliferative diseases (MPDs).



**Figure 1. Cellular response of HSCs to various stresses.** HSCs possess a specialized intracellular machinery that allows them to respond to various cellular stresses. The degree of stimulation, along with the function of the intracellular response pathways, determines HSC cell fate.

## AGING PHENOTYPE OF THE HEMATOPOIETIC SYSTEM

In principle, HSCs can persist and continue to divide for periods longer than the host organism's lifespan; in practice, however, various aging-related phenomena can alter their functions and limit their survival. The hematopoietic system undergoes a variety of age-related changes (Figure 2). The most prominent phenotype of an aged hematopoietic system is the skewing of differentiation potential toward the myeloid lineage (Beerman et al., 2010; Sudo et al., 2000). Recently, it was shown that this lineage bias of the hematopoietic system can be attributed to biased clonality in HSCs that arises as a function of age (Beerman et al., 2010). When long-term repopulating HSCs (LT-HSC: lineage<sup>-</sup>Sca1<sup>+</sup>Kit<sup>+</sup>flt3<sup>-</sup>CD34<sup>-</sup>) were divided into CD150<sup>high</sup> and CD150<sup>low</sup> subsets, the CD150<sup>high</sup> population exhibited a higher myeloid differentiation potential and a higher self-renewal advantage (Beerman et al., 2010). Consequently, the CD150<sup>high</sup> population gradually comes to dominate the stem cell pool at later ages. The clonal expansion of HSC populations with myeloid lineage bias may explain why the incidence of myeloid neoplasms increases with age. Another feature of an aged hematopoietic system is the increase in the absolute number of HSCs (Morrison et al., 1996b; Sudo et al., 2000). In mice, limiting dilution analysis revealed a two-fold increase in the number of multi-lineage repopulating cells in BM of aged mice, relative to the number in young mice (Sudo et al., 2000). Past observations from serial BM transplantation experiments had suggested that the function of HSCs from aged donors were equivalent to HSCs from young donors (Harrison et al., 1978; Ogden and Mickliem, 1976). Recently, however, more stringent assays have revealed that the competitive repopulation potential of HSCs from aged donor mice is inferior to that of



**Figure 2. Aging process of HSCs.** HSCs are exposed to multiple intrinsic and extrinsic cellular stresses, which cause various intracellular changes and deterioration of the niche. These changes cause the aging-related phenotypes of the hematopoietic system.

HSCs from young mice (Chambers et al., 2007). When identical numbers of HSCs (side population cells, which are Lineage<sup>-</sup> Sca-1<sup>+</sup>, Kit<sup>+</sup>), from either old or young mice, were competitively transplanted into lethally irradiated mice, the HSCs from old mice displayed a lower repopulation potential at 8 and 16 weeks after transplantation (Chambers et al., 2007); old HSCs had approximately three-fold lower long-term repopulation potential. These results suggested that the increase in HSCs numbers might serve to compensate for their functional defects (Chambers et al., 2007). Furthermore, HSCs from aged mice exhibit altered homing and mobilization properties (Morrison et al., 1996b; Xing et al., 2006). Such an increase in the number of phenotypically defined HSCs is also revealed in humans by quantification of lineage<sup>-</sup>, CD34<sup>+</sup>, CD38<sup>-</sup>, CD90<sup>+</sup> cells within the BM (Pang et al., 2011). However, it is not known whether human HSCs develop similar functional defects with age.

Some aspects of aging in the hematopoietic system can be attributed to aging at the cellular level, in particular cellular senescence. Cellular senescence is a state in which a cell irreversibly loses its capacity to divide, i.e., undergoes permanent growth arrest, in response to various stresses (Campisi, 2001). The cellular senescence response normally converges on two established pathways involving the tumor suppressor proteins cyclin-dependent kinase inhibitor 2A (Cdkn2a, Ink4a/Arf, p16<sup>Ink4a</sup>-p19<sup>Arf</sup>)<sup>Ink4a</sup> and transformation related protein p53 (Trp53) (Campisi, 2001).

## Ink4a and Arf

Ink4a and Arf are tumor suppressor proteins encoded by the *Cdkn2a* (*Ink4a/Arf*) locus (Krishnamurthy et al., 2004). Ink4a and Arf expression levels are elevated in multiple types of senescent cells both *in vitro* and *in vivo*; (Alcorta et al., 1996; Kiyono et al., 1998; Krishnamurthy et al., 2004; Randle et al., 2001) thus, Ink4a and Arf expression levels are considered biological markers of senescence (Krishnamurthy et al., 2004). Ink4a causes hypophosphorylation of retinoblastoma tumor suppressor protein (Rb) by inhibition of cyclin dependent kinase 4 and 6 (CDK4/6), resulting in suppression of E2f transcription factor (E2f)-dependent gene expression and formation of repressive heterochromatin (senescence-associated heterochromatic foci (SAHF)), which blocks cell cycle progression from G1 to S. (Campisi, 2005; Sharpless and DePinho, 1999) Trp53 and the cyclin-dependent kinase inhibitor 1A (P21) act upstream of Ink4a activation, yet cell-cycle changes caused by Ink4a are permanent and cannot be reversed by Trp53 inactivation (Beausejour et al., 2003). Immature HSCs (Lin<sup>-</sup>Kit<sup>+</sup>Sca1<sup>+</sup>CD34<sup>low</sup>Flk-2<sup>low</sup> cells) from aged mice exhibit increased *Ink4a* mRNA expression compared to young mice (Janzen et al., 2006). HSCs from old *Ink4a/Arf*<sup>-/-</sup> mice exhibit higher reconstitution potential in competitive

BM transplantation (Janzen et al., 2006). Moreover, Ink4a is upregulated in the context of premature senescence of HSCs in many genetically manipulated mice models. For example, HSCs in mice lacking the Ewing sarcoma breakpoint region 1 gene (*Ewsr1<sup>-/-</sup>*), a causative gene for malignant bone tumors, lose quiescence, decrease in number and exhibit enhanced expression of Ink4a (Cho et al., 2011). The relationship between cellular senescence and aging of HSCs, however, has not been fully resolved; in a recent study, more than 99% of aged HSCs failed to express *Ink4a/Arf* at the mRNA level, and senescence-associated histone methylation profiles involving the *Ink4a/Arf* locus were not observed in aged HSCs (Attema et al., 2009). Furthermore, the downstream target of Ink4a, Rb, is dispensable for steady-state hematopoiesis (Daria et al., 2008). Rb interacts with E2Fs (E2f1, E2f2, and E2f3) and controls cell proliferation and differentiation through the regulation of the G1-S transition at the beginning of the cell cycle (Talluri and Dick, 2012). HSCs from *Vav1-Cre/Rb<sup>fl/fl</sup>* mice exhibit normal steady-state functions, but have reduced long-term repopulation potential during serial BM transplantation, demonstrating the relevance of Rb in stress hematopoiesis (Daria et al., 2008). Although the role of Ink4a in intrinsic HSC senescence has been questioned, it is clear that impaired Ink4a function in the microenvironment promotes senescence in hematopoiesis (Oguro et al., 2006). In contrast to Ink4a, Arf alone appears to be dispensable for HSC regulation: *Arf*-null HSCs do not exhibit any repopulation advantage during competitive BM transplantation (Stepanova and Sorrentino, 2005).

## Trp53

The tumor suppressor protein Trp53 is a transcription factor that is activated upon cellular stress to induce cell-cycle arrest (Mercer et al., 1990), senescence (Serrano et al., 1997), or apoptosis (Shaw et al., 1992). The significance of Trp53 in hematopoiesis has been well documented (Kastan et al., 1991; Lotem and Sachs, 1993). LT-HSCs express high levels of Trp53 (Dumble et al., 2007), and HSCs from mice deficient in Trp53 exhibit a lower fraction of cells in quiescence (Liu et al., 2009b). Trp53 expression is increased in LT-HSCs deficient in the oncogene *Elf4/Mef* (ETS domain transcription factor (Liu et al., 2009b)); *Elf4/Mef* recruits Mdm2 (transformed mouse 3T3 cell double minute 2), the ubiquitin ligase that degrades Trp53 at steady-state (Ringshausen et al., 2006; Sashida et al., 2009). Trp53 functions are associated with those of *Elf4/Mef*, as demonstrated by the fact that Trp53 deficiency can reverse the enhanced quiescence of *Elf4/Mef*-deficient HSCs (Liu et al., 2009b). P21 is the major target of Trp53, yet P21 deletion in HSCs resulted in only minimal changes in HSC functions (van Os et al., 2007). Gene expression profile analysis of Trp53-null HSCs identified Gfi1 (growth factor independent 1) and Necdin as possible targets of Trp53

in HSCs (Liu et al., 2009b). Experiments in which *Gfi1* and *Necdin* were deleted have implicated them as positive regulators of HSC self-renewal and functional integrity (Hock et al., 2004; Taniura et al., 2005; Taniura et al., 1999). Furthermore, knock-in mice expressing an active truncated form of *Trp53* (*Trp53<sup>+/m</sup>*) exhibit a premature senescence phenotype associated with a decline in HSC number (Maier et al., 2004; Tyner et al., 2002) and a disadvantage in engraftment upon transplantation (Dumble et al., 2007).

## **REACTIVE OXYGEN SPECIES (ROS)**

A large fraction of the energy production in a eukaryotic cell depends on aerobic metabolism in the mitochondria. Energy production in the mitochondria uses molecular oxygen and is accompanied by generation of ROS (Balaban et al., 2005). ROS include multiple oxygen-containing molecules, including superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. It has been proposed that high ROS levels promote cellular senescence, apoptosis, and carcinogenesis (Balaban et al., 2005; Harman, 1956). Endogenous ROS are produced primarily at two sites, the cytochrome I and III complexes of the mitochondria, where electrons are sometimes prematurely released during cytochrome-catalyzed reactions (Balaban et al., 2005). Exogenous sources of ROS include irradiation and administration of radiomimetic chemicals (Ames and Shigenaga, 1992).

ROS levels are related to cell fate (Macip et al., 2003). A cell experiences approximately  $2 \times 10^4$  DNA-damaging events per day, and a majority of these events are caused by ROS (Ames and Shigenaga, 1992). In addition to their harmful effects, however, ROS can also mediate beneficial effects: ROS levels are utilized by the cell to regulate cell fate decisions regarding survival and proliferation (Chen and Pervaiz, 2007, 2009); thus, at physiological levels, ROS mediate signals that are vital for cells. Nonetheless, overproduction of ROS is detrimental, and cells have acquired pathways to eliminate excessive ROS: superoxide is degraded by mitochondrial manganese superoxide dismutase (*Sod2*, *MnSOD*), and hydrogen peroxide is eliminated by glutathione peroxidase (*Gpx*), periaxin (*Prx*,) or catalase (*Cat*) (Cohen and Hochstein, 1963; Pervaiz et al., 2009; Remacle et al., 1992).

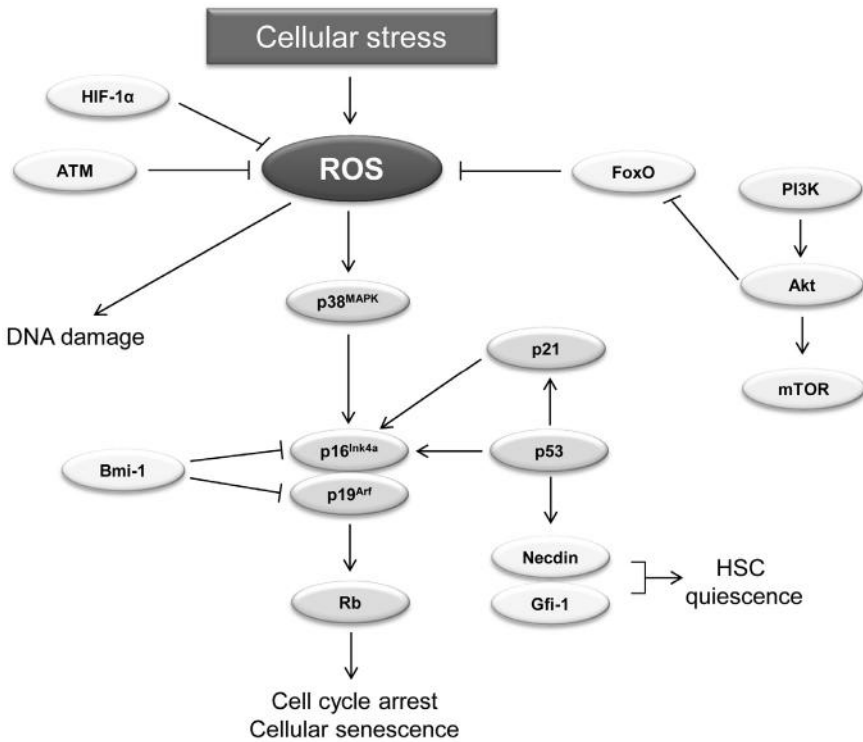
HSCs residing in the adult BM exist in a hypoxic state and preferentially utilize anaerobic glycolysis pathways for energy production (Takubo et al., 2010). HSCs are thought to be more vulnerable than other cells to ROS accumulation (Suda et al., 2011), and the hypoxic microenvironment is an important means of protecting them from oxidative stress. ROS levels in HSCs are correlated with HSC functions. For example, when ROS levels of LT-HSCs (*CD34<sup>-</sup>*, *Lin<sup>-</sup>*, *Sca-1<sup>+</sup>* and, *Kit<sup>+</sup>*) were measured using dihydrodichlorofluorescein diacetate (DCF-DA) staining, DCF<sup>low</sup> LT-HSCs exhibited higher reconstitution and self-renewal potentials

than DCF<sup>high</sup> LT-HSCs, both *in vivo* and *in vitro* (Jang and Sharkis, 2007). Upon transplantation and engraftment, the differentiation pattern of the DCF<sup>high</sup> LT-HSCs exhibited myeloid skewing, mimicking the lineage bias observed in aged mice. The functional defects of DCF<sup>high</sup> LT-HSCs could be pharmacologically rescued by administration of an antioxidant (NAC), a Mapk14 (mitogen-activated protein kinase 14, p38) inhibitor, or an inhibitor of Mtor (rapamycin) (Jang and Sharkis, 2007). These observations indicate that ROS accumulation negatively regulates LT-HSC functions. However, changes in ROS levels may influence determination of cell fate (Chen and Pervaiz, 2009; Saretzki et al., 2004). Indeed, in a recent study of *Drosophila* hematopoietic cells, the hematopoietic progenitor population displayed high ROS levels, and scavenging ROS from these cells reduced their differentiation potential (Owusu-Ansah and Banerjee, 2009). Furthermore, reducing ROS levels through the administration of NAC decreases the number of LT-HSCs (CD34<sup>+</sup>, Lin<sup>-</sup>, Sca-1<sup>+</sup> and, Kit<sup>+</sup>) capable of engrafting to the BM of lethally irradiated mice, indicating that BM homing is dependent on high ROS levels (Lewandowski et al., 2010). Together, the observations described in this section demonstrate that ROS levels can have varying effects on cell fate in HSCs.

## ROS Regulators and HSC Aging

Because intracellular ROS levels control the homeostasis and function of HSCs, disturbances in pathways that regulate ROS production are associated with premature senescence (Figure 3).

The protein kinase ataxia telangiectasia-mutated (Atm) participates in a DNA-damage response pathway that regulates apoptosis and cell-cycle checkpoint control in response to DNA double-strand breaks (DSBs), telomere erosion, and oxidative stress (Shiloh, 2003). Stem cells from Atm-deficient mice undergo premature senescence (Takubo et al., 2008). In humans, defects in the Atm-gene cause ataxia telangiectasia, a disorder associated with signs of premature aging (McKinnon, 2012). Cells lacking Atm exhibit high concentrations of ROS and hypersensitivity to agents that induce oxidative stress. Atm-deficient mice contain lower numbers of HSC subsets, and these cells exhibit a disadvantage in repopulation potential during competitive BM transplantation (Ito et al., 2004). *Atm*<sup>-/-</sup> mice develop BM hypoplasia with advanced age (Ito et al., 2004). The defects in HSCs in *Atm*<sup>-/-</sup> mice cannot be rescued by telomerase reverse transcriptase (Tert) overexpression, indicating that the phenotypes of *Atm*<sup>-/-</sup> HSCs are not a consequence of telomere dysfunction. On the other hand, *Atm*<sup>-/-</sup> HSCs exhibit high concentrations of ROS, and their functional defects can be rescued upon NAC administration (Ito et al., 2004). Atm-related HSC phenotypes can also be rescued by overexpression of Ink4a. Furthermore,



**Figure 3. Regulation and effects of ROS in HSCs.** Over-accumulation of ROS in HSCs is prevented by the action of several proteins, including Atm, Hif1a, and Foxo. High levels of ROS activate the Ink4a/Rb pathway, resulting in cell-cycle arrest and cellular senescence.

accumulation of ROS activates Mapk14, which upregulates Ink4a, thereby inducing senescence (Ito et al., 2006; Serrano et al., 1997). These data indicate that Atm functions to suppress abnormal ROS production in order to sustain HSC self-renewal.

Another group of critical regulators of ROS production in HSCs, identified shortly after Atm, is the Foxo family of Forkhead homeobox transcription factors. In mammals, the Foxo family comprises Foxo1, Foxo3, Foxo4, and Foxo6 (Greer and Brunet, 2005). Foxo3-deficient HSCs exhibit abnormally high accumulation of ROS due to defects in the induction of antioxidant enzymes. Disturbed quiescence and self-renewal of HSCs in Foxo3<sup>-/-</sup> mice result in reduction of their repopulation potential; consequently, Foxo3a<sup>-/-</sup> mice develop severe loss of HSCs in the BM as a function of age (Greer and Brunet, 2005). Furthermore, mice bearing conditional deletions of three Foxo family members, Foxo1/3/4, under control of the *Mx-Cre* system, exhibit a decrease in HSC number and repopulation potential. Similar to Foxo3<sup>-/-</sup> HSCs, the compound deletion



of *Foxo1/3/4* in HSCs results in aberrant accumulation of ROS. Genetic profiling of *Foxo1/3/4<sup>-/-</sup>* HSCs revealed a significant upregulation of ROS leading edge genes, which overlapped with genes associated with the transition of HSCs to myeloid progenitors. These findings implicate Foxo genes in the regulation of ROS production, which are in turn involved in the regulation of HSC differentiation (Tothova et al., 2007). Foxo is regulated through phosphorylation by activated Akt (Storz, 2011). Phosphorylated Foxos are exported from the nucleus to the cytoplasm, where they lose the ability to activate transcription (Brunet et al., 1999). To maintain Foxo3a in the nucleus, the PI3K/Akt pathway is suppressed in HSCs during steady-state hematopoiesis (Miyamoto et al., 2007; Yamazaki et al., 2006). AKT1/2 double-deficient HSCs from fetal livers exhibit defective differentiation capacity along with impaired long-term reconstitution potential (Juntilla et al., 2010). AKT1/2 doubly-deficient HSCs also exhibit accumulation of intracellular ROS (Juntilla et al., 2010).

The hypoxia-activated protein hypoxia inducible factor 1 alpha (Hif1a) also regulates ROS production in steady-state hematopoiesis (Takubo et al., 2010). Hif1a null mice exhibit a loss of HSC quiescence and diminished hematopoietic functions with aging. Defects in Hif1a-null HSCs are dependent on Ink4a expression, and can be restored by suppressing Ink4a by transducing a retroviral Bmi1 expression vector. Hif1a-null HSCs express high levels of ROS, which are thought to cause HSC defects (Takubo et al., 2010).

## **Telomere Elongation and DNA Damage Response**

The intrinsic replicative life span of a cell is determined by telomere length. Upon critical telomere shortening, cell division arrests at the G1 phase (Campisi et al., 2001). Telomeres include repetitive DNA sequences and proteins that form a cap at chromosome ends; the caps protect chromosomes from degradation or fusion (Campisi et al., 2001). Telomerase is a ribonucleoprotein complex that elongates telomeres, thereby contributing to chromosome stability and maintenance of genomic integrity (Collins, 2000). In most somatic lineages, telomerase is active during embryonic development but downregulated after birth, and is present at barely detectable levels throughout the rest of the life span (Blasco et al., 1995). By contrast, telomerase is expressed in HSCs, and telomerase activity is essential for the maintenance of HSC self-renewal capacities (Morrison et al., 1996a). Colony assays of BM cells from telomerase-deficient mice (*Terc<sup>-/-</sup>*) revealed a significant decrease in the number of colonies formed from late generations of *Terc<sup>-/-</sup>* mice compared to early generations (Lee et al., 1998). In serial BM transplantation experiments, HSCs from late-generation *Terc<sup>-/-</sup>* mice also exhibit diminished repopulation capacities (Samper et al.,

2002). However, over-expression of *Tert* in HSCs does not enhance HSC transplantation capacities in serial BM transplantation (Allsopp et al., 2003). We recently revealed an additional role of *Tert* in HSC regulation by reporting that *Atm* and *Tert* double-deficient mice exhibit premature aging and shorter overall lifespan than *Atm*-null mice (Nitta et al., 2011). Premature aging in the *Atm/Tert* double-knockout mice was dependent on *Ink4a* and *ARF*, but not on *P21* (Nitta et al., 2011). The mechanism underlying the premature aging of *Atm/Tert* double-knockout mice was attributed to a higher vulnerability of HSCs to ROS mediated apoptosis (Nitta et al., 2011). Transplantation of young HSCs into old recipient mice or *Terc*<sup>-/-</sup> mice revealed a defect in HSC engraftment and B-lymphopoiesis compared to young recipient mice (Ju et al., 2007). Furthermore, when BM cells from *Terc*<sup>-/-</sup> mice were transplanted into young wild-type recipients, no alterations in B-lymphopoiesis or myelopoiesis were observed (Ju et al., 2007). These studies underscored the importance of telomerase function in the niche that supports HSCs.

Both intrinsic and extrinsic cellular stresses cause accumulation of DNA damages. Cells possess DNA damage response pathways that detect and repair DNA damage, but when these pathways fail, accumulation of damage may result in cell cycle arrest (senescence), apoptosis, or uncontrollable proliferation (malignancy) (Warren and Rossi, 2009). Numerous pathways involving DNA repair have been associated with HSC aging. Rossi et al. previously showed that endogenous DNA damage accumulates with age in wild-type HSCs (Rossi et al., 2007a). Although accumulated DNA damage did not deplete HSC pools with age, stem cell functional capacity was severely affected under conditions of stress, leading to loss of reconstitution and proliferative potential, diminished self-renewal, increased apoptosis, and ultimately functional exhaustion (Rossi et al., 2007a). Several DNA damage response pathways have been elucidated by studies of the pathophysiology of progeroid syndromes. For example, the causative gene for ataxia telangiectasia, *Atm*, is required for DNA repair (Shiloh, 2003). Defects in nucleotide excision repair pathways result in xeroderma pigmentosum (XPD) (DiGiovanna and Kraemer, 2012). Genetic manipulation of these genes in mice exhibit their functional significance in HSCs. Mice bearing mutations in genes related to DNA repair pathways, such as non-homologous end joining (NHEJ) (*Xrcc5/Ku80*<sup>-/-</sup>) or nucleotide excision repair (*Ercc2/XPD*<sup>TTD</sup>), exhibit defects in HSC functions that worsen with age: reduction in the number of lymphoid progenitors, diminished repopulation capacities, defective self-renewal, and enhanced apoptosis (Rossi et al., 2007a; Warren and Rossi, 2009). Together, the accumulation of DNA damage in HSCs, coupled with the down-regulation of damage repair pathways, underscores the important role played by declining genome integrity in HSC aging.

## EPIGENETIC REGULATION

One difficulty in studying aging is that the phenotypic outcome of aging varies among individuals. Changes in the epigenetic profiles of HSCs may cause such large variations. Epigenetic regulations are defined as influences on the gene expressions of the cell without interfering with the primary DNA sequences (Jaenisch and Bird, 2003). Epigenetic regulations involve DNA methylation, histone modifications and changes in the chromatin structure (Konuma et al., 2010). Recent reports have shown that HSC differentiation and lineage commitment is highly influenced by DNA methylation (Bock et al., 2012). Genome-wide expression profiling on HSCs purified from old and young mice showed that genes associated with lymphoid specification are downregulated with age, whereas those associated with myeloid specification were upregulated (Rossi et al., 2005). Furthermore, expression levels of many proto-oncogenes are increased as a function of aging (Rossi et al., 2005). By contrast, another study comparing the gene expression profiles of old and young HSCs reported that the transcriptional profiles of old and young HSCs are similar, suggesting that the transcriptional program of HSCs is not broadly altered with age (Chambers et al., 2007). In that study, aged HSCs exhibited downregulation of specific genes involved in transcriptional silencing via chromatin regulation, such as the SWI/SNF-related chromatin remodeling genes (*Smarca4* and *Smarb1*), histone deacetylases, and a DNA methyltransferase (*Dnmt3b*) (Chambers et al., 2007). Together, these findings suggest global dysregulation of transcriptional activity in aged HSCs.

Polycomb group (PcG) proteins form transcriptional repressor complexes that regulate histone modifications (Rajasekhar and Begemann, 2007). PcG proteins have been shown to play a central role in HSC maintenance (Konuma et al., 2010). B cell-specific Moloney MLV insertion site-1 (*Bmi1*) is a polycomb group (PcG) protein, which acts epigenetically by repressing gene transcription through histone modifications (Sauvageau and Sauvageau, 2010). *Bmi-1* was one of the first molecules to be linked to HSC senescence. *Bmi-1* is expressed at high levels in immature hematopoietic cells (Schuringa and Vellenga, 2010), and HSCs from *Bmi-1*-deficient mice undergo premature senescence, exhibit BM hypoplasia, and reduction in HSC number; upon transplantation, these HSCs exhibit only a transient repopulation potential (Lessard and Sauvageau, 2003; Park et al., 2003). At the molecular level, *Bmi-1* deficient HSCs exhibit elevated levels of *Ink4a* and *Arf* expression (Park et al., 2003). Overexpression of *Ink4a* and *Arf* in *Bmi-1*<sup>-/-</sup> HSCs induces apoptosis, whereas the functional deficiencies of *Bmi-1*<sup>-/-</sup> HSCs are rescued by deletion of *Ink4a* and *Arf* (Oguro et al., 2006). Forced expression of *Bmi-1* in HSCs enhances their symmetrical cell division and a marked increase of HSC repopulation potential *in vivo*,

demonstrating the relevance of Bmi-1 to HSC self-renewal (Iwama et al., 2004). Furthermore, Bmi-1 also regulates mitochondrial ROS production (Liu et al., 2009a). *Bmi-1*<sup>-/-</sup> thymocytes exhibit increased production of ROS in association with enhanced DNA repair pathways (Liu et al., 2009a). These reports show that Bmi-1 is a crucial epigenetic regulator that prevents HSC aging through the repression of Ink4a and Arf signals. The epigenetic regulation of HSCs along with linking various external stimuli to specific epigenetic changes is an emerging field in the study of HSCs.

## THE AGING NICHE

HSCs in the adult BM reside in a specialized microenvironment termed “the niche”, which influences their quiescence, self-renewal, proliferation, and differentiation (Schofield, 1978). The HSC niche is structured by the non-hematopoietic cells in the BM; these cells interact with HSCs through adhesion and through secretion of various humoral factors (Arai et al., 2005). The importance of the niche on the physiological homeostasis of HSCs has been clearly established, eliminating the possibility that age-related changes of HSCs are solely cell-autonomous. However, although the intrinsic regulation of HSC aging has been extensively studied, investigations of alterations in the niche and their association with aging-related HSC phenotypes have not progressed as far. Subcutaneous implantation of BM and spleen stroma from aged and young mice show that hematopoietic cells engrafted onto stroma from old mice exhibited less efficient repopulation than cells engrafted onto young stroma (Hotta et al., 1980). A study using time-lapse two-photon microscopy to visualize aged and young hematopoietic stem and progenitor cells (HSPCs) engrafting in the BM revealed changes in the interactions between aged HSCs and the niche; specifically, aged HSPCs engrafted distal to the periosteal region of the BM, and showed less cellular polarity (Kohler et al., 2009). A striking feature of the aged BM microenvironment is the prominent increase in adipocytes (Naveiras et al., 2009). Lipoatrophic Tg(AZIP/F)1Vsn mice, which are genetically incapable of adipocyte formation, exhibit an increase in HSC engraftment after irradiation (Naveiras et al., 2009). Furthermore, two cholesterol efflux regulators, the adenosine triphosphate-binding cassette (ABC) transporters Abca1 and Abcg1, inhibit HSPC proliferation, as does high-density lipoprotein (HDL); these observations link HSC function to cholesterol metabolism (Yvan-Charvet et al., 2010). A precise characterization of the aging niche is still necessary, as is a deeper understanding of the functional significance of the interactions between the niche and aged HSCs.

## CONCLUSION

HSC functions are tightly regulated, both by cell-autonomous mechanisms and through interactions with their microenvironment. The accumulation of various cellular stresses converges on HSC aging, during which HSCs lose their ability to self-renew and govern the homeostasis of the hematopoietic system. We have focused on the underlying mechanisms involving aging of HSCs, specifically focusing on ROS. Ongoing research on aging HSCs continues to identify the pathways involved in the progression of the aging process, and to delineate the aging niche. Further studies aimed at elucidating the mechanisms involved in HSC aging may provide advances in regenerative medicine and inspire novel treatment strategies for age-related hematological diseases.

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## ABBREVIATIONS

|       |                                                      |
|-------|------------------------------------------------------|
| MDS   | myelodysplastic syndrome                             |
| MPD   | myeloproliferative diseases                          |
| SAHF  | senescence-associated heterochromatic foci           |
| ROS   | reactive oxygen species                              |
| DSB   | double strand break                                  |
| XPD   | xeroderma pigmentosum                                |
| NHEJ  | non-homologous end joining                           |
| HSPCs | hematopoietic stem and progenitor cells              |
| ABC   | adenosine triphosphate-binding cassette transporters |

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# Bioelectric Controls of Stem Cell Function

*Vaibhav P. Pai and Michael Levin\**

## SUMMARY

The field of stem cell biology has gained momentum over the past decade and half due to its potential to cure damaged and diseased tissues and shed light on the processes by which a single fertilized egg gives rise to the remarkable complexity of the embryonic body. Hopes for biomedical applications are fueled by the detection of resident adult stem cell populations in almost all tissues, including the central nervous system. The majority of efforts to understand and control the cues that regulate embryonic stem cell differentiation into various tissues have focused on biochemical signals and transcriptional networks. However, bioelectric signals mediated by slow changes in ion flows and transmembrane voltage gradients encode patterning information in physiological networks that guide embryonic morphogenesis, regeneration, and cancer suppression. Recent molecular work has begun to unravel the mechanisms of endogenous bioelectric influences on stem cell function. Here, we discuss fundamental

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*List of abbreviations after the text.*

properties of bioelectric signals, the mechanisms by which they regulate stem cell maintenance and functional differentiation, and the prospects of manipulation of such signals for therapies in regenerative medicine.

## INTRODUCTION

### Definitions and Brief History

Stem cells are distinctly unspecialized cells with unique features of self renewal and the ability to differentiate along various cell lineages. Three most common types of stem cells are the embryonic stem cells derived from inner mass of the embryo (ES cells), somatic stem cells, and induced pluripotent stem cells (iPS) (Jaishankar and Vrana, 2009; Kao et al., 2008). Stem cell maintenance and differentiation requires continuous signal exchange among the stem cells and their surrounding microenvironment (niche). Alongside well-known biochemical signals exists an important and fascinating system of *bioelectrical* communication. These signals are mediated by endogenous ion flows, electric fields, and voltage gradients that ultimately derive from the action of ion channels and pumps; they are generated, and likely received, by all cells. These endogenous information-bearing bioelectrical events are distinct from the high amplitude, very rapid action potentials of excitable nerve and muscle. In contrast, developmental bioelectric signals derive from steady-state currents and resting membrane potentials and their characteristic time scale is measured in hours to days. Many reviews have been written about endogenous bioelectric signals especially in wound healing and cell motility (Kenneth and Robinson, 1996; McCaig et al., 2009; Nuccitelli, 2003a; Zhao, 2009), as well as embryogenesis (Adams and Levin, 2012a; Levin, 2009, 2012). While most of the work on the mechanisms by which bioelectric cues control cell behavior has involved differentiated somatic cells, roles for instructive ion flows have begun to be uncovered in ES cells as well. Here we review available data on the bioelectric signals involved in maintaining stem cells' niche and driving their differentiation.

Earliest discoveries of bioelectric signals can be traced as far back as Luigi Galvani in 1700's. A number of modern workers used functional electrophysiology techniques (Borgens, 1982, 1988; Borgens et al., 1983; Hotary and Robinson, 1994; Jaffe and Nuccitelli, 1977; Jaffe and Stern, 1979; Marsh and Beams, 1946a, b, 1947, 1952; Nuccitelli and Erickson, 1983; Nuccitelli et al., 1977; Nuccitelli et al., 1986; Robinson, 1985; Robinson and Messerli, 2003) to show that bioelectric signals are broadly conserved from plants (Cortal et al., 2008; Feijo et al., 2001) to mammals (Borgens,

1999; Borgens et al., 1987; Borgens et al., 1994). Such ion-based signals control crucial aspects of cell behavior regulation including proliferation, apoptosis, differentiation, shape, orientation, and migration. Classical studies demonstrate endogenous bioelectric signals as bearers of important instructive information guiding limb regeneration (Jenkins et al., 1996), tail development (Hotary and Robinson, 1992), cell migration and orientation through the embryo (Borgens and Shi, 1995; Shi and Borgens, 1995), and oogenesis (Woodruff and Telfer, 1980) in a range of amphibian, avian, and invertebrate model systems.

With the rise of molecular biology, emphasis was placed on those questions best addressed using the state-of-the-art techniques, which are most suitable for probing biochemical events. As a result, most of the effort in the field of stem cell regulation has been focused on such biochemical signals. Importantly, bioelectrical gradients are far more than housekeeping processes: they are carriers of instructive signals controlling individual cell behavior and the orchestration of cell functions within the context of a host organism (Levin, 2007; Levin, 2009, 2012; McCaig et al., 2005; McCaig et al., 2009). Thus, an understanding and mastery of bioelectrical signals will significantly extend our ability to regulate stem cell maintenance and differentiation and are exciting targets for regenerative medicine.

Unlike the electrical circuits, in which current consists of electron flows, bioelectric signals are carried by charged ions like sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), chloride ( $\text{Cl}^-$ ), and protons ( $\text{H}^+$ ); calcium ( $\text{Ca}^{2+}$ ) is usually excluded because it signals by virtue of its chemical nature, at levels far too low to affect transmembrane potential. Such ion fluxes are generated by ion channels and pumps within cell membranes (this review will focus mostly on gradients at the cell plasma membrane, although similar events occur at organelle membranes as well). The resulting separation of charge across the membrane gives rise to transmembrane voltage potential ( $V_{\text{mem}}$ ) (usually inside negative  $-30$  to  $-70$  mV). The different aspects of spatially-distributed voltage gradients (electric fields, individual ion fluxes, and  $V_{\text{mem}}$  potentials) can all carry developmental information at significant distances, as can passive properties of gap junctions which can regionalize tissues into iso-electric cell groups (Cooper, 1984; Fitzharris and Baltz, 2006; Zimmermann et al., 2009).

Thus spatiotemporal patterns of resting potential and ion flux are known to participate in the patterning signals that coordinate cellular activity towards the construction and maintenance of anatomy. Recent technological advancement and proof-of-principle applications of high-resolution functional studies of bioelectric controls now make it possible to study the mechanisms and relevance of these signals for regulation of stem cell activity.

## Modern Tools for Detection and Manipulation of Bioelectric Signals

Understanding the roles of biophysical parameters (pH,  $V_{\text{mem}}$ , ion flux) will enhance our ability to specifically direct stem cells in the direction of the damaged or injured tissue for its replacement and restoration of function. A significant first step in this direction is the ability to map physiological properties of differentiating stem cells *in vitro*. Much progress in this direction has been made in the area of stem cell-derived cardiomyocytes (SC-CM). For functional restoration it is essential that SC-CMs must electrically couple to host cardiac tissue. Electrophysiological mapping techniques like microelectrode-arrays and optical mapping are employed (Weinberg et al., 2010). Microelectrode-arrays record extracellular measurements at numerous points across a small area (0.01–0.25 cm<sup>2</sup>) to assess electrical propagation. This set-up allows multiple recordings over space and time (Binah et al., 2007; Reppel et al., 2004). Microelectrode-arrays can also be used as high-throughput assays to test pharmacological drugs in terms of their effect, specificity and safety and efficiency (Caspi et al., 2009; Meyer et al., 2004). Optical mapping uses fluorescent labels (most common are  $V_{\text{mem}}$  and intracellular calcium [ $\text{Ca}^{2+}$ ], sensitive dyes) that can be applied to areas of different sizes (Efimov et al., 2004; Entcheva et al., 2000; Salama and Morad, 1976). These techniques can be used to detect waves of electrical activation in heart, neural and other stem cells (Leao et al., 2010; Li et al., 2008b). Similar to microelectrode-arrays, optical mapping can also be used for high-throughput screens. New fluorescent dyes reveal a variety of cellular ion flows, i.e.: [ $\text{Na}^+$ ], magnesium [ $\text{Mg}^{2+}$ ], potassium [ $\text{K}^+$ ], pH, and these techniques can be applied to monitoring the slowly changing bioelectrical properties of stem cells and their non-excitable progeny (Moschou and Chaniotakis, 2000; Steinberg et al., 2007; Wolff et al., 2003; Yun et al., 2007). Additional methods now available include highly sensitive ion-selective extracellular electrode probes (Reid et al., 2007; Reid and Zhao, 2011a; Reid and Zhao, 2011b; Smith et al., 2007) and nano-scale voltage reporters (Tyner et al., 2007). Further, various specific strategies for quantitatively characterizing *in vivo* physiology have now been established (Adams and Levin, 2012a; Adams and Levin, 2012b, 2012a; Oviedo et al., 2008a; Weinberg et al., 2010).

Importantly, a plethora of reagents and methodologies have recently been developed for functional analysis of bioelectric signals with molecular specificity (Adams, 2006a; Adams, 2008). Inverse drug screen can be performed (Adams and Levin, 2006) to implicate specific ion transporter proteins in any process of interest. This is most often used to probe endogenous bioelectrical mechanisms and has resulted in the identification of channels and pumps as novel components of stem cell

regulation (Morokuma et al., 2008; Oviedo and Levin, 2007b; Sundelacruz et al., 2008). Targeted misexpression of hyperpolarizing and depolarizing channels in cells of interest now offers the opportunity to specifically perturb the  $V_{\text{mem}}$  in any cell of interest, characterizing the resulting phenotype to understand the role of bioelectric cues in pattern formation in a range of tractable model systems (Beane et al., 2011; Blackiston et al., 2011; Levin et al., 2002; Pullar et al., 2006; Pullar and Isseroff, 2005; Rajnicek et al., 2006; Tseng et al., 2010). Such reagents make the molecular dissection of the transduction and transcriptional response steps possible that link biophysical and physiological events to downstream changes in cell behavior and anatomy.

A powerful new family of techniques, optogenetics, makes use of light gated ion channels (such as opsins) that control  $V_{\text{mem}}$  and thus allow great spatio-temporal resolution for functional perturbations of voltage gradients (Gradinaru et al., 2010; Lin, 2011). In one such study channelrhodopsin-2-yellow fluorescent protein was stably introduced into mES cells, which were then sorted by fluorescence activated cell sorting (Stroh et al., 2011). Inward current was induced by blue light pulses. These mES cells retained the capability to differentiate into functional mature neurons as assessed by action potentials, fast excitatory synaptic transmission and expression of neuronal proteins. Automation of such processes allows precise chronic and temporal, non-invasive optical control of ES cells both *in vitro* and *in vivo*; it is likely that as optogenetic tools evolve to encompass reagent for control of resting potential, not only rapid action potentials, the technology will be incorporated into scaffolds and other bioengineered platforms for increased control of stem cell behavior via patterned light-regulated bioelectric modulation.

A great variety of new tools to map and control bioelectrical properties are now coming available. These tools will allow researchers to chart the bioelectric properties of stem cells to understand the interplay between physiological and genetic regulators of stem cell function *in vivo*.

## **THE ROLE OF BIOELECTRICITY IN GUIDING STEM CELL DIFFERENTIATION AND MAINTAINING THE NICHE DURING EMBRYOGENESIS**

Significant endogenous voltage gradients and electric fields have been documented in avian, amphibian, and mammalian embryos that are important for organ patterning (Hotary and Robinson, 1994; McCaig and Robinson, 1982; Metcalf and Borgens, 1994; Robinson and Messerli, 2003). The spatio-temporal patterns of these gradients are non-uniform across the embryo and are hypothesized to hold the necessary information required for

embryonic stem cell development and regeneration (Hotary and Robinson, 1992; Metcalf and Borgens, 1994). A cell's resting  $V_{\text{mem}}$  is a result of imbalance in the ionic concentrations that accumulate along the cell membrane. Thus, externally-applied electric fields have been used to redistribute ions, thus superimposing  $V_{\text{mem}}$  changes upon the resting  $V_{\text{mem}}$  (Pucihar et al., 2009). The kinetics and amplitudes of  $V_{\text{mem}}$  induced by externally applied electric pulses can be estimated analytically using Laplace's or Schwan's equation (Lojewska et al., 1989; Pucihar et al., 2009). The calculated  $V_{\text{mem}}$  responses to the applied electric field have been corroborated using direct experimental measurements. Significant hyperpolarization and depolarization can result from even millisecond application of electric fields of physiological strength 10–100 mV/cm.

Application of such external electric fields is now used as a tool to study the responses of embryonic stem and multipotent cells to bioelectrical differentiation cues.

## **Embryoid Cells Driven Towards Vasculature via Bioelectric Cues**

Embryonic bodies derived from mES cells when exposed to pulsed electric fields showed increased vascular endothelial growth factor (VEGF) expression (Sauer et al., 2005). Similar results were also observed in cultured human umbilical cord stem cells (Zhao et al., 2004). VEGF is a potent angiogenic factor and in turns triggers proliferation, migration, and remodeling of matrix which are necessary for angiogenesis (Ferrara, 1995; Matsunaga et al., 2008). The exact mechanism of electric field-mediated VEGF stimulation is not known but a role of reactive oxygen species in this process has been postulated (Sauer et al., 2005). Bioelectrical control of vascular growth may be a useful addition to the current toolkit for treatment of vascular diseases including coronary artery diseases (by induction of new vessels) or by limiting or redirecting tumor vasculature.

## **Bioelectric Cues in Directing Embryonic Neural Stem Cells (NSC) Growth and Differentiation**

Endogenous electric fields have been shown to be central for embryonic neural development and in damaged and regenerating nervous system. Steven Ingvar in 1920 showed for the first time that chick embryonic neural explants orient the nerve processes in presence of electric fields (7mV/mm) (Ingvar, 1920). Avian neurite fibers were deflected towards the cathode and growth on cathode side was much faster (Marsh and Beams, 1946a, b). Disaggregated *Xenopus laevis* embryonic NSC have become a great



tool for studying this effect as it allows studying single cells eliminating the possibility of explants movement. The developing *Xenopus* neural tube is easily accessible; the neurons are easy to grow and are robust and relatively large. It also allows direct comparison with the chemotaxis data that are usually generated using *Xenopus* preparations. These *Xenopus* preparations, in well-controlled experimental conditions for electrode material and induced chemical gradients in the media, also showed a strong preference towards current sink (cathode) (Hinkle et al., 1981; McCaig et al., 2005). Such effects of electric fields on embryonic neural directionality have also been shown in chicks and rodents (Jaffe and Poo, 1979; Rajnicek et al., 1992). These observations have had important consequences for clinical efforts to stimulate central nervous system (CNS) repair following injury (Borgens, 1999; Borgens et al., 1987). Similar observations have been made on embryonic derived muscle cells, neural crest cells, fibroblasts and epithelial cells (Robinson, 1985). A major caveat for these studies is the observation that different culture conditions (media components, culture surface coating and charge) dramatically affect the response of the cultured neurons making it difficult to predict how the neurons would react *in vivo* (McCaig et al., 2000; Rajnicek et al., 1998). Hence it is key to understand the bioelectric physiology and environment of these embryonic precursor cells *in vivo* in order to be able to duplicate these conditions to guide and direct them towards specific lineages *in vitro*.

### **Gap Junctional Communication in Maintaining Stem Cells During Embryonic Development**

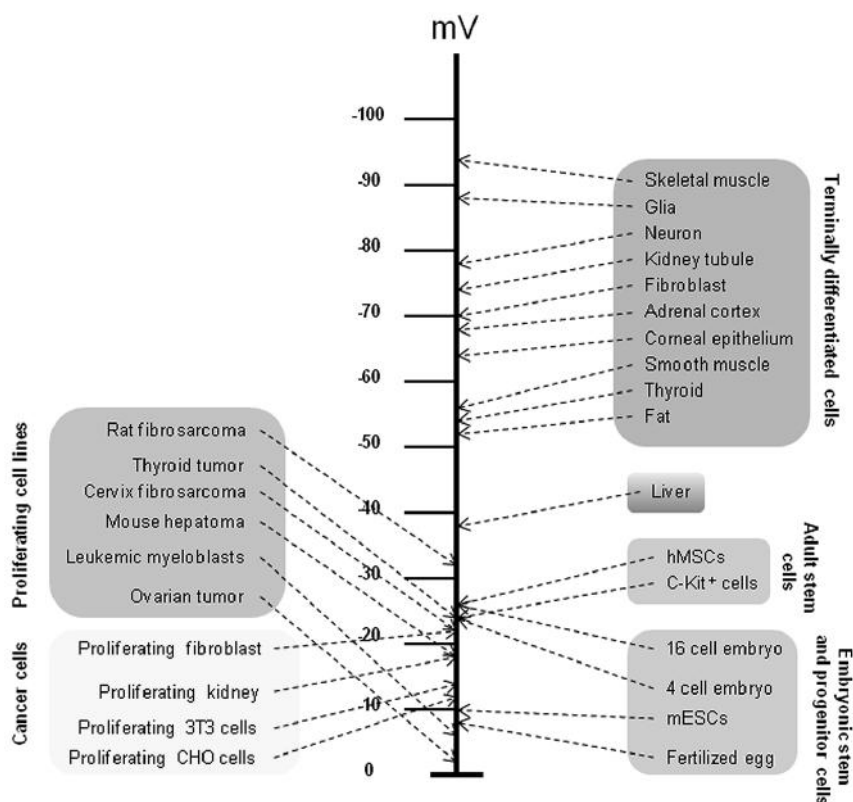
Gap junctional channels (GJC) enable direct passage of water-soluble small molecule signals from the cytoplasm of one cell to that of its neighbor, bypassing the normal routes of transmembrane secretion. This is an extremely versatile system of communication between cells, allowing rapid synchronization among cells and the passage of signals, both of which can be regulated at the level of gap junction number, permeability state, selectivity, etc. (Falk, 2000; Goodenough and Musil, 1993; Goodenough and Paul, 2009; Herve et al., 2007; Mese et al., 2007). GJCs are a perfect conduit for developmental information flow, which depends on the ability of cells and tissues to communicate, or restrict communication in specific instances, to allow proper differentiation (Rela and Szczupak, 2004; Sutor and Hagerty, 2005). The control of spatial spread of bioelectric signals by GJC paths among cells in any tissue allows efficient, coordinated regulation of cell migration, proliferation, and differentiation (Levin, 2003; McCaig et al., 2005; Nuccitelli, 2003a; Nuccitelli, 2003b; Robinson and Messerli, 2003).

A fine balance of proliferation and differentiation must be maintained in populations of stem cells of the embryo that support dynamic maintenance of organ systems. Gap junctions are beginning to be uncovered as an element of this process (Sheardown and Hooper, 1992; Tazuke et al., 2002; Trosko, 2005; Trosko et al., 2000; Wong et al., 2004). Direct dye coupling assays have demonstrated compartments of bioelectric signal communication via GJC in a number of embryonic systems, such as squid (Potter et al., 1966), newt (Ito and Loewenstein, 1969), chick (Sheridan, 1966), and frog (Guthrie et al., 1988; Guthrie, 1984; Levin and Mercola, 1998, 2000; Nagajski, 1989; Turin and Warner, 1980). Interestingly, NSC exhibit a unique signature based on GJC and ion transporters, and GJC are essential for their survival and proliferation (Cai et al., 2004). Germ line stem cells differentiate upon losing contact with their niche, which likely involves GJC.

Direct physiological communication through gap junctions allows regulated propagation of bioelectrical signals across cell fields; it is likely to be an important component of maintaining the stem cell niche.

## Role of $V_{\text{mem}}$ in Embryonic Stem Cell Maintenance and Differentiation

It has long been observed (Binggeli and Weinstein, 1986) that cellular  $V_{\text{mem}}$  well-correlated with overall proliferation and plasticity/differentiation state (Figure 1). High degree of polarization (hyperpolarized  $V_{\text{mem}}$ ) tends to be present in differentiated quiescent cells, whereas low polarization (depolarized  $V_{\text{mem}}$ ) is associated with mitotically active progenitor or stem-like cells.  $V_{\text{mem}}$ -associated changes have been shown to regulate proliferation and differentiation in embryonic precursor/stem cells, with a major role of  $K^+$  current regulation (Park et al., 2007; Wang et al., 2005). In human and mouse ES cells, delayed outward rectifying  $K^+$  current ( $IK_{\text{DR}}$ ) (depolarize  $V_{\text{mem}}$ ) is present and is permissive for proliferation, as application of specific  $K^+$  channel blockers inhibited DNA synthesis (Wang et al., 2005). In *Xenopus* embryos, the  $K^+$  channel Kcnq1 (Kv7.1) contributes significantly to the membrane potential (Morokuma et al., 2008). Misexpression of its regulatory subunit Kcne1 (Mink, Isk), suppressed Kcnq1 currents, resulting in  $V_{\text{mem}}$  depolarization and ectopic induction of the neural crest regulator genes *Sox10* and *Snai2* (*Slug*). The result was over-proliferative melanocytes with a highly invasive migratory phenotype resembling metastasis (Morokuma et al., 2008). Surprisingly, some studies show that depolarization of mES cells by blocking Kv channels prevents proliferation and induces differentiation in these cells (Ng et al., 2010). Although, Kv channels have been shown to be present in mES cells (Wang et al., 2005) their endogenous activity is not yet determined as depolarization would be essential in the first place to activate



**Figure 1.  $V_{mem}$  control of stem cell maintenance and differentiation.** Physiological measurements of various cell types as documented in [modified from (Binggeli and Weinstein, 1986). Stem cells and highly proliferative cells have a relatively depolarized  $V_{mem}$ . However, quiescent, terminally differentiated cells are strongly polarized. A deviation from this is seen in the liver tissues which have a  $V_{mem}$  closer to the proliferative and stem cells than differentiated cells. It highlights the highly responsive progenitor cells population and high regenerative ability of liver tissues. Analogously, proliferative cancer cells also cluster with stem and highly proliferative cells. The relationship between  $V_{mem}$  and cell fate is thus a functional one, e.g., mature CNS neurons were induced to reenter cell cycle by forced depolarization (Stillwell et al., 1973) and not a secondary manifestation of cell fate (Levin, 2003).

*Color image of this figure appears in the color plate section at the end of the book.*

these channels. Nonetheless, the potential utility of  $V_{mem}$  as a controller of cell behavior is demonstrated by experiments where post-mitotic CNS neurons can be coerced into reentering cell cycle by long term depolarization (Cone and Cone, 1976; Cone and Tongier, 1971; Stillwell et al., 1973). A fascinating aspect of these studies was that, the effect was maximal at 100  $\mu$ M of drug blocking  $K^+$  current and declined at 1 mM (Yasuda et al., 2008). Such biphasic effects suggest the presence of an optimal membrane potential range—window effect—resulting in further higher order information storage

and signals within different ranges of cell's  $V_{\text{mem}}$ . These data reveal  $V_{\text{mem}}$  as a bearer of important instructive information guiding stem cells of the embryo along different lineages. A thorough understanding of how differentiation information is encoded in cell-autonomous and microenvironment-driven bioelectric signals during embryonic development will provide essential information for guidance of stem cells along desired lineages.

## **BIOELECTRICITY DEFINES THE STEM CELLS NICHE DURING TISSUE REGENERATION AND HOMEOSTASIS DURING ADULTHOOD**

Repairing tissues lost due to injury, age, or disease is one of the prime goals of stem cells biology and regenerative medicine. The potential of regenerative systems has been recognized for more than a century (Morgan, 1901). However some of the fundamental questions still remain (Birnbaum and Sanchez Alvarado, 2008; Goss, 1969). How does a system know it is injured and when repair should stop? What is the nature of the information that governs organ structure? Is regeneration a manifestation of resident multipotent cells or transdifferentiation? Are the programs used for regeneration co-opted from developmental programs or are there specific injury related programs present within the system (Borgens, 1984)? Is regeneration inherent to living systems or is it an adaptation? Can physiological control points be used to enhance and guide regeneration?

A major effort towards this goal is to understand the cues that guide cell behavior during morphogenesis with the hopes that it might answer some of these questions. Biochemical means of manipulating stem cells has been the mainstream approach but data collected over the past century indicate that bioelectric signals (ion flows,  $V_{\text{mem}}$ ) constitute an important system of cellular control (Levin, 2009; McCaig et al., 2009).

### **Neural Stem Cell Differentiation**

#### ***Voltage gradients affecting neurogenesis***

Endogenous voltage gradients have been shown to be present in embryos of various species, notably in the developing nervous system (Burr and Bullock, 1941; Burr and Hovland, 1937; Hotary and Robinson, 1992, 1994; Shi and Borgens, 1994, 1995). One of the first pieces of evidence for voltage gradients affecting neuronal growth in the mammalian CNS was from embryonic rat hippocampal neurons in culture (Rajnicek et al., 1992). The axons were observed to be orthogonally aligned to the applied voltage gradient vector and it was suggested that the endogenous voltage gradients

may control the structural and functional polarity of CNS neurons. Neural progenitor cells have now been identified in the spinal cord and mature brain and are recruited to the site of injury (Hawryluk and Fehlings, 2008; Jin et al., 2006; Zhao et al., 2008). Various types of brain damage like trauma, stroke, and degenerative disorders stimulate both, the generation of endogenous electrical signals and the proliferation and differentiation of endogenous neural progenitors (Elliott et al., 2003; Fallon et al., 2000; Gould and Tanapat, 1997; Madhavan et al., 2009; Magavi et al., 2000; McCaig et al., 2009; Yamamoto et al., 2001). Electric fields can regulate the axis of neural cell division *in vivo* (Borgens and Shi, 1995; Sausedo et al., 1997; Tuckett and Morriss-Kay, 1985) and guide neuronal migration (Li et al., 2008a; Meng et al., 2011). Hence, a combination of NSC transplants and electrical fields to guide their migration and differentiation has been proposed (Yao et al., 2011). However, the high complexity of nervous system structure and function brings significant technical challenges to direct control of electric field-mediated neurogenesis. Functional biomaterials like hydrogels, silk films with conductive properties, and nanotechnology are potentially promising solutions to these problems (Cho and Borgens, 2010; Cho et al., 2009; Kim et al., 2010; Kim et al., 2009; Matos and Cicerone, 2010). Clinical application in regulating injured spinal cord neuron regrowth via applied voltage gradients are now being performed (Borgens, 1999; Shapiro et al., 2005).

Thus, voltage gradients serve as conduits of important bioelectric information not only during embryonic neural development, but also during neurogenesis from adult stem cells. However, extensive technological development is necessary before this information can be tapped into for biomedical applications.

### ***V<sub>mem</sub> in regulating adult neural stem cell niche***

In the adult mammalian neurogenic niche of the sub-ventricular zone (SVZ), self-renewing and multipotent NSC give rise to actively proliferating cells called 'transit-amplifying cells', followed sequentially by neuroblasts (Doetsch et al., 1999; Garcia et al., 2004; Imura et al., 2003; Laywell et al., 2000; Morshead et al., 2003; Seri et al., 2001). NSC are mixed together with and transit-amplifying cells that are more generally called neural precursor cells (NPC). The electrical properties of adult NSC/NPC cell membranes are characterized by a hyperpolarized resting  $V_{\text{mem}}$  ( $\sim -75\text{mV}$ ) (Liu et al., 2006; Yasuda et al., 2008). Unlike adult NPC, neonatal and embryonic NPC have a more depolarized  $V_{\text{mem}}$  between  $\sim -55\text{mV}$  and  $\sim -40\text{mV}$  respectively (Cai et al., 2004; Cesetti et al., 2009; Nguyen et al., 2002; Owens et al., 1996; Smith et al., 2008), consistent with the long-known relationship between depolarization and degree of differentiation (Cone, 1970, 1971; Cone and

Tongier, 1971). Also, adult NPC exhibit predominantly delayed rectifying  $K^+$  ( $IK_{DR}$ ) currents with minor or no contribution from rapidly inactivating  $K^+$  ( $IK_A$ ) currents (Liu et al., 2006; Yasuda et al., 2008). However, in neonatal NPC,  $IK_{DR}$  and  $IK_A$  currents of almost equal amplitude are observed (Cesetti et al., 2009) (see Table 1 and 2). The difference in the  $V_{mem}$  and bioelectric properties of neonatal and adult NPC may reflect the morphological observation that the neonatal SVZ undergoes a developmental shift from an embryonic neurogenic niche and is distinguishable from the adult.

In adult NPC, the hyperpolarized  $V_{mem}$  is attributed to high  $K^+$  conductance of the resting membrane (Yasuda et al., 2008), which is due to the presence of  $Ba^{2+}$ -sensitive inwardly rectifying  $K^+$  ( $K_{ir}$ ) channels (Liu et al., 2006; Yasuda and Adams, 2010; Yasuda et al., 2008). Sub-millimolar  $Ba^{2+}$  and increased extracellular  $K^+$  concentration elicits depolarization to  $\sim -55$  mV and enhances growth factor-stimulated NPC proliferation (Yasuda et al., 2008), thus suggesting that  $V_{mem}$  depolarization is the underlying mechanism of controlling NPC proliferation. Analogously, during astrocyte cell cycle progression from G1/S checkpoint to S phase, there is a transient depolarization associated with decreased  $K_{ir}$  channel activity (MacFarlane and Sontheimer, 2000). Such cell cycle progression and proliferation can be induced in astrocytes by sub-millimolar  $Ba^{2+}$  mediated decrease in  $K_{ir}$  channel activity causing depolarization. Similarly, the NPC cell cycle is thought to be regulated by  $K_{ir}$  channel activity. Several different  $K_{ir}$  channel isoforms have been identified in adult NPCs in the SVZ (Liu et al., 2006; Yasuda et al., 2008). Hence it has been hypothesized that in adult NPC,  $K_{ir}$  channels may act as a sensor detecting changes in  $K^+$  concentration in extracellular space, cerebrospinal fluid or plasma  $K^+$  levels during normal and pathological conditions (Yasuda and Adams, 2010).

Interestingly, further depolarizing the  $V_{mem}$  to  $\sim -30$  mV with higher concentration of  $Ba^{2+}$  decreases the NPC proliferation. This suggests the presence of an optimal  $V_{mem}$  window for NPC proliferation. Such a non-linear relationship between  $V_{mem}$  and proliferation has been shown in mammalian cells (Sundelacruz et al., 2009) and a similar window was recently found for the voltage range specifying embryonic cells towards an eye morphogenesis fate (Pai et al., 2012). Thus,  $V_{mem}$  serves as a powerful control knob for regulating adult stem cells and their niche. More importantly, they provide a mode of manipulating stem cell behavior using pharmacological means that do not require genetic manipulation.

### **Gap junctions in regulating adult neural stem cell niche**

Gap junctions are known to play various physiological roles, providing electrical and metabotropic intercellular communication because of their capability to pass various aqueous small molecules  $< 1$  kDa, such as cations,

**Table 1.** Mammalian stem cells and bioelectric signals regulating their specific paths.

| Species         | Cell type            | Channel /current expressed                 | Bioelectric property             | Phenotype                     | Modification of property                                                                      | Effect                             | Phenotype                           | References                                  |
|-----------------|----------------------|--------------------------------------------|----------------------------------|-------------------------------|-----------------------------------------------------------------------------------------------|------------------------------------|-------------------------------------|---------------------------------------------|
| Human           | Fibroblast           |                                            |                                  |                               | ClC-3 Cl <sup>-</sup> channels                                                                | Differentiation, hyperpolarization | Myo-fibroblasts                     | (Yin et al., 2008)                          |
| Human           | Umbilical cord cells |                                            |                                  |                               | External pulsed field                                                                         | VEGF expression                    |                                     | (Zhao et al., 2004)                         |
| Human and Mouse | ESCs                 | IK <sub>DR</sub>                           | Depolarized V <sub>mem</sub>     | Proliferation                 | K <sup>+</sup> channel blocker                                                                | Inhibited DNA synthesis            |                                     | (Wang et al., 2005)                         |
| Mouse           | ESCs                 |                                            |                                  |                               | External pulsed fields                                                                        | VEGF expression                    |                                     | (Sauer et al., 2005)                        |
| Mouse           | NPCs                 | K <sub>ir</sub>                            | Hyper-polarized V <sub>mem</sub> | Quiescence                    | Ba <sup>2+</sup> inhibition of K <sub>ir</sub>                                                | Depolarize V <sub>mem</sub>        | Proliferation                       | (Yasuda et al., 2008)                       |
| Mouse           | ESCs                 |                                            |                                  |                               | Transient outward K <sup>+</sup> channel, Na <sup>+</sup> , K <sub>ir</sub>                   | Hyperpolarization                  | Differentiation into cardio-myocyte | (van Kempen et al., 2003)                   |
| Mouse           | carcinoma P19 cells  |                                            |                                  |                               | K <sub>ir</sub> , transient outward K <sup>+</sup> channel, Na <sup>+</sup> , K <sub>ir</sub> | Hyperpolarization                  | Differentiation into cardio-myocyte | (van der Heyden et al., 2003)               |
| Mouse           | C2C12 cells          | K <sub>ATP</sub> , Cl <sup>-</sup> current |                                  | Proliferative state           | Tetradotoxin sensitive Na <sup>+</sup> , IK <sub>DR</sub> , K <sub>ir</sub>                   | Differentiation                    | myoblasts                           | (Fioretti et al., 2005; Voets et al., 1997) |
| Mouse           | Cortex NSCs          | V-ATPase                                   |                                  | Stem cell state maintenance   | Inhibition of H-v-ATPase                                                                      |                                    | Differentiation into neurons        | (Lange et al., 2011)                        |
| Mouse           | Astrocyte            | Decreased K <sub>ir</sub>                  | De-polarization                  | G <sub>1</sub> /S progression | Ba <sup>2+</sup> inhibition of K <sub>ir</sub>                                                | Enforce Depolarization             | Enforce proliferation               | (MacFarlane and Sontheimer, 2000)           |

anions, nutrients, metabolites and, second messengers (Dbouk et al., 2009). A marked increase in expression of connexin (Cx) 43 gap junctional protein has been observed between the neonatal period and adulthood in the SVZ (Miragall et al., 1997) and NSC in the adult SVZ exhibit coupling to 4–10 other NSC (Liu et al., 2006; Liu et al., 2005). This reflects the postnatal development of the adult neurogenic niche (Kong et al., 2008; Liu et al., 2006).

During rat embryonic development, GJCs have been shown to be essential for NSC' survival and proliferation (Cai et al., 2004). Cx43 and Cx45 were found in dissociated embryonic rat NSC. The presence of functional GJCs was confirmed via small fluorescent dye spreading after microinjection into single NSC (Cai et al., 2004). Further, pharmacological disruption of GJCs resulted in inhibited proliferation and death of these NSC. Similar observations were made in embryonic mouse cortical NSC, where Cx43 mediated gap junctions were essential for survival and proliferation (Cheng et al., 2004; Duval et al., 2002). Inhibition of GJCs resulted in either death or differentiation of the NSC, while Cx43 overexpression promoted self-renewal (Elias and Kriegstein, 2008).

In adult mammals, Cx43 and Cx30 are expressed in hippocampal radial glia-like NSC where they are required for NSC proliferation and neurogenesis (Kunze et al., 2009). Mice lacking Cx30 and Cx43 in these NSC showed significant decline in NSC and almost complete inhibition of proliferation. Similar observations were made using inducible virus-mediated Cx ablation, which reduced neurogenesis (Kunze et al., 2009). The central canal of the spinal cord has also been shown to support a niche of spinal NPC, which in some vertebrates are responsible for functional recovery after spinal cord injury. These cells show a high density of Cx43 and whole-cell patch clamp showed extensive dye coupling of cells, suggesting strong GJC coupling among the NPC (Russo et al., 2008). Thus, the spinal NPC share basic gap junctional properties of embryonic and adult brain neurogenic niche. Understanding such aspects may help tap into the potential for generation of new neurons *in situ* for cell replacement therapies.

Interestingly, Cx expression and GJC coupling is lost in both embryonic and adult NSC as they differentiate into neuronal precursors. These neural precursor cells then become refractory to the GJC blockers (Cai et al., 2004; Kunze et al., 2009). All this strongly suggest that Cx and GJC communication are required for neurogenesis in embryonic as well as adult brain.

What molecules actually flow through GJCs in mediating signals that maintain the NSCs niche remains to be fully investigated. One role for gap junctions in bioelectrical signaling is to demarcate iso-potential cell groups and average out neighboring cells'  $V_{\text{mem}}$  levels. The NSCs in adult mice SVZ, with a hyperpolarized  $V_{\text{mem}}$ , Cx43 expression and GJC (assayed via dye



spreading) show a passive current profile (Liu et al., 2006). They maintain whole cell currents that are mainly due to electrical currents through GJC. Similar observations have been made in the hippocampal radial glia-like NSC (Bahrey and Moody, 2003; Lo Turco and Kriegstein, 1991). In goldfish retinal marginal zone (retinal adult stem cell niche), the progenitor cells are coupled by GJCs with large amount of passively flowing ion current between them (Tamalu et al., 2001). Their differentiation however, is accompanied by junctional uncoupling. In addition, it is also possible that the GJC is an important downstream target–gap junctions–regulated by transjunctional voltage (Harris et al., 1983; Spray et al., 1981; Verselis et al., 1997) and the cells'  $V_{\text{mem}}$  levels may be important in regulating the passage of other, non-electrical signals among the cells.

The above set of data indicates that the electrical signals coupling the NSC via GJCs is essential for their survival and proliferation; loss of this ability accounts for their differentiation.

### ***Ion flux changes as markers of stem cell differentiation stages***

Two key questions must be answered in order to extract therapeutically relevant information about  $V_{\text{mem}}$  in stem cell differentiation: (1) what are the electrophysiological differences between the differentiated and undifferentiated states, and (2) are these differences instructive for differentiation? The majority of work has been done in neural and muscular systems, as the acquisition of electrophysiological features contributes to the excitability of the mature cell. Comprehensive physiomics profiling is needed to formulate quantitative models that explain the bioelectrical differences among cells at different steps of differentiation. During early stages of development, maturing neural crest cells express human *ether à gogo*-related genes encoding  $K^+$  currents ( $I_{\text{HERG}}$ ) and  $IK_{\text{DR}}$  currents, while during later stages, neural crest cells exhibit  $V_{\text{mem}}$  hyperpolarization and expression of  $IK_{\text{DR}}$ ,  $IK_{\text{IR}}$ , and  $Na^+$  ( $I_{\text{Na}}$ ) currents (Arcangeli et al., 1995; Arcangeli et al., 1998; Arcangeli et al., 1997; Arcangeli et al., 1999). Based upon the observed physiological remodeling, it was suggested that the ordered expression of ion channels defines neural crest cell developmental stages (Arcangeli et al., 1997). Characteristic changes in  $Na^+$  and  $K^+$  channel expression and the induced ionic currents have also been found to accompany neural differentiation of other stem-like cell types, such as neural stem-like cells from human umbilical cord blood (Sun et al., 2005), immortalized hNSC (Cho et al., 2002), and mESC (Chafai et al., 2006) (see Table 1 and 2). Thus, electrophysiological profiles can be coupled with traditional immunocytochemical techniques to describe the maturation

state of cells and to distinguish among different cell populations originating from common precursors.

Crucially, electrophysiological changes play functional, instructive roles in the differentiation process and hence provide more than a passive readout of developmental stage or of lineage commitment. Neural differentiation has been shown to depend on the function of specific ion transporters such as the  $\text{Na}^+/\text{K}^+$ -ATPase (Messenger and Warner, 1976, 1979), vacuolar  $\text{H}^+$  ATPase (Lange et al., 2011), and voltage-gated  $\text{Na}^+$  channels (Pineda et al., 2005; Pineda et al., 2006; Ribera and Nüsslein-Volhard, 1998; Svoboda et al., 2001). Hyperpolarization has been shown to influence the development and maturation of mammalian cerebellar granule cells with the hypothesis that these  $V_{\text{mem}}$  changes alter  $\text{Ca}^{2+}$  signaling to control stage-specific gene expression (Nakanishi and Okazawa, 2006; Rossi et al., 1994). Analogously, developmentally-upregulated genes were downregulated with depolarization, while developmentally-downregulated genes were upregulated with depolarization (Sato et al., 2005). This suggests that the physiological state ( $V_{\text{mem}}$ ) has a powerful control over the precursor cell genetics.

Overall these experiments suggest that the changes in specific ion fluxes are not only differentiation markers of stem cell progeny but by their influence on overall  $V_{\text{mem}}$ , actually participate in regulating the stem cell differentiation process.

## **Planarian Neoblast Differentiation**

The flatworm planaria is a bilaterally symmetrical animal with three tissue layers, multiple organ systems, and a complex brain and CNS. It also possesses impressive powers of regeneration (Reddien and Sanchez Alvarado, 2004) making it a powerful model for the study of the control of stem cells in the context of large-scale pattern formation. A worm can be cut into many pieces, and each piece can regenerate into a complete worm. The basis of this ability is the presence, throughout the animal, of adult stem cells called neoblasts. When a worm is bisected, the cells at the anterior-facing edge of the first fragment must form a head, while the cells at the posterior-facing edge of the other fragment form a tail. These cells were adjacent neighbors before the cut and yet implement completely different morphological structures, making this an excellent system in which to study the relationship between large-scale anatomical form and stem cell behaviour. In order to know what structures remain in any given fragment after injury (and thus recreate only what is missing), the blastema cells must receive information from the remaining tissues. Since this involves information transfer over several cell lengths, the role of GJC in this process has been investigated (Oviedo and Levin, 2007a).

Meticulous inhibition of each of the 12 GJC transcripts expressed in the planaria showed that Smedinx11 is required for normal regeneration and proper neoblast niche maintenance and function (Oviedo and Levin, 2007a, b; Reddien and Sanchez Alvarado, 2004). Moreover, Smedinx11 is also required for proliferation and maintenance of the stem cell population. While it is now known that  $V_{\text{mem}}$  (Beane et al., 2011) and GJC (Nogi and Levin, 2005) are both key determinants of anatomical identity during planarian regeneration, much work remains to flesh out the mechanisms by which GJC-permeable signals and  $V_{\text{mem}}$  interact to specify patterning signals for neoblast progeny. In particular it is not yet known whether GJC is used to establish cell fields of specific  $V_{\text{mem}}$  regions, or whether voltage gradients regulate the movement of small molecule chemical morphogens through GJC paths during regenerative morphogenesis.

## **Mesenchymal Stem Cells (MSC) Differentiation**

### ***Electrophysiological properties***

The developmental electrophysiology of myocyte differentiation has been characterized. Differentiation of mouse embryonic stem cells (van Kempen et al., 2003) and embryonic carcinoma P19 cells (van der Heyden et al., 2003) into cardiomyocytes correlates with up-regulation of cardiac-related ion channels in specific temporal patterns. Transient outward  $K^+$  channels are up-regulated early during differentiation, and  $Na^+$  and delayed inward rectifier  $K^+$  channels later during differentiation (van der Heyden et al., 2003). Skeletal myoblasts also exhibit distinct current and ion channel profiles in their proliferating and differentiating states.

Murine C2C12 myoblasts undergoing active proliferation express an ATP-induced  $K^+$  current and a swelling activated  $Cl^-$  current (Fioretti et al., 2005; Kubo, 1991; Voets et al., 1997). Upon initiation of differentiation, these currents are replaced with a tetrodotoxin-sensitive  $Na^+$  current, and  $IK_{\text{DR}}$  currents (Kubo, 1991; Lesage et al., 1992; Voets et al., 1997; Wieland and Gong, 1995). From these data it appears that the change in the profile of  $K^+$ ,  $Cl^-$  and  $Na^+$  current is an essential aspect of MSC proliferation and differentiation. A significant amount of physiomic characterization remains to be performed before data mining can extract highly-specific, predictive signatures for different cell states.

### ***$V_{\text{mem}}$ mediated modulation of MSC***

Several recent studies have demonstrated that endogenous  $V_{\text{mem}}$  modulation is an instructive signal during MSC differentiation and maturation. For

example,  $V_{\text{mem}}$  hyperpolarization precedes human myoblast differentiation and is essential for this process since myocyte fusion and transcription factor activity are blocked when hyperpolarization is blocked (Konig et al., 2006; Konig et al., 2004; Liu et al., 1998). Similarly, expression of the chloride channel  $\text{ClC3}$  and its corresponding  $\text{Cl}^-$  current is required for fibroblast-to-myofibroblast differentiation (Yin et al., 2008) and expression and function of two inward  $\text{K}^+$  rectifier channels is essential for the differentiation of human hematopoietic progenitor cells (Shirihai et al., 1998; Shirihai et al., 1996). Since endogenous hyperpolarization occurs upstream of known conventional biochemical signaling events also hints at the possibility of using a single control point to modulate the differentiation-related signaling pathways.

$V_{\text{mem}}$  controls hMSC differentiation decisions *in vitro* (Sundelacruz et al., 2008). hMSC ( $V_{\text{mem}} -40\text{mV}$ ) undergo hyperpolarization during both osteogenic ( $V_{\text{mem}} -56\text{mV}$ ) and adipogenic ( $V_{\text{mem}} -74\text{mV}$ ) differentiation (Sundelacruz et al., 2008). When normal  $V_{\text{mem}}$  progression was disrupted by forced depolarization, suppression or delay of both osteogenic and adipogenic differentiation was observed. Conversely, during osteogenic differentiation, treatment with hyperpolarizing drugs induced upregulation of bone-related gene expression indicating accelerated differentiation (Sundelacruz et al., 2008). These observations provide compelling evidence for an instructive role of  $V_{\text{mem}}$  in differentiating hMSC. Interestingly, bioelectric cues can overcome competing biochemical signals; for example depolarization trumps the induction of differentiation by insulin and dexamethasone in hMSC (Sundelacruz et al., 2008) while physiological-strength electric fields override opposing chemical trophic factors, contact inhibition release, and population pressure during wound healing (Zhao, 2009). Future possibilities include maintenance of a renewable stem cell population *in vitro* and acceleration or augmentation of stem cell differentiation for therapeutic purposes or for tissue engineering, if scaffold and bioreactor constructs are designed in a way that allows pharmacological and genetic manipulation of  $V_{\text{mem}}$  (Hechavarria et al., 2010).

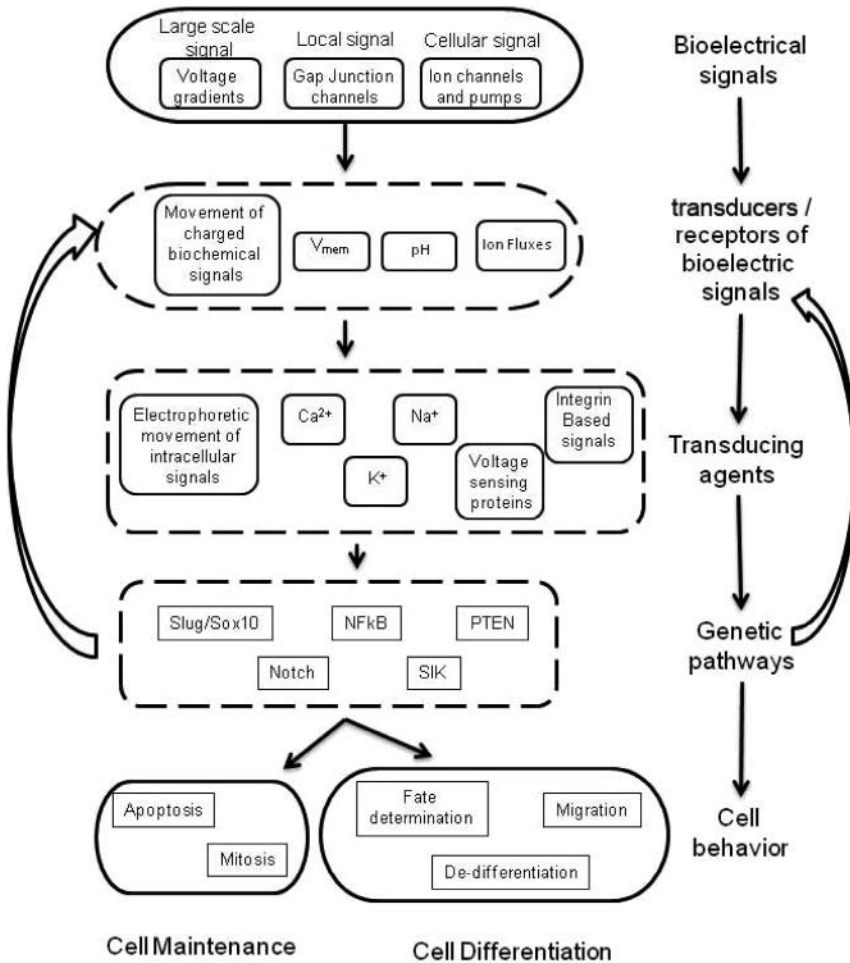
The data generated so far clearly suggest that bioelectric signals are an integral part of defining the adult stem cell niche in several tissues (e.g., neural and mesenchymal). Moreover, changes in ion channel expression/function, the resulting ion fluxes, and ultimately  $V_{\text{mem}}$  gradients, form a very important instructive signal for the stem cells to proliferate and differentiate along specific lineages. This bioelectric control of stem cell fate regulation is conserved between invertebrate (planaria) to vertebrate systems (amphibians).

## Mechanism of Bioelectric Signal Transduction

Bioelectrical signals (carried by the spatio-temporal pattern of ion flows through ion channels, pumps, and gap junctions) are found both upstream and downstream of conventional biochemical and genetic pathways (Figure 2). Expression of ion translocator proteins is under genetic and biochemical control and the bioelectrical signals themselves can influence downstream biochemical and genetic signals. Misexpression of channel and pump mutants can be used in gain- and loss-of-function approaches to specifically modulate different aspects of ion flux, *in vivo* or *in vitro* (Adams and Levin, 2012a). For example, misexpression of electroneutral transporters can differentiate between  $V_{\text{mem}}$  change vs. flux of specific ions as being the information-bearing signal. Pore mutants can distinguish between ion conductance roles vs. possible functions of channels/pumps as scaffolds or binding partners (nonelectrical signaling). These reagents, coupled with more standard molecular-genetic and biochemical readouts of cell state (e.g.: mRNA expression analyses) are being used to flesh out the amplification and transduction pathways through which biophysical events couple to transcriptional outcomes and thus control stem cell behavior.

Specialized sensory cells can distinguish external electric fields as weak as 5 nV/cm (Kalmijn, 1971, 1982). The most common mechanism linking  $V_{\text{mem}}$  change and downstream events is calcium influx (via voltage-sensitive  $\text{Ca}^{2+}$  channels) (Sasaki et al., 2000; Shi et al., 2000; Zayzafoon, 2006). Most of  $\text{Ca}^{2+}$  signaling events take place through specialized receptors such as calmodulin or calcineurin (Konig et al., 2006). Other  $V_{\text{mem}}$ -transducing mechanisms include voltage-dependent changes of integrin conformation involving ERG1 channels (Arcangeli and Becchetti, 2006; Cherubini et al., 2005). Recent evidence suggests that integrins can regulate ion channels and form macromolecular complexes, resulting in localization of channels onto plasma membrane microdomains. Such integrin-channel complexes regulate downstream signaling like tyrosine kinase and GTPase. Additional mechanisms that transduce bioelectrical signal into second-messenger cascades include modulation of the activity of voltage-sensitive small-molecule transporters (e.g., the serotonin transporter and GJCs, which converts  $V_{\text{mem}}$  into the flux of specific chemical signals) (Levin et al., 2006).

An exciting recent discovery involves voltage sensitive phosphatase, a phosphoinositide phosphatase that converts  $\text{PI}(3,4,5)\text{P}_3$  to  $\text{PI}(4,5)\text{P}_2$  in a manner regulated by a voltage sensor domain (Murata et al., 2005). The identification of a protein able to transduce  $V_{\text{mem}}$  into all of the potential downstream pathways controlled by this powerful second-messenger



**Figure 2. Interaction and integration of bioelectrical signals with biochemical and genetic pathways.** Bioelectrical signals include large scale signals like voltage gradients and electric fields, local signals via gap junction channels and cellular level signals via functional changes in ion channels and pumps. These bioelectrical signals are perceived/received by the cells in terms of change in their  $V_{mem}$ , pH, specific ion flows, movement of charged biochemical signal. Intracellularly, these signals are transduced by voltage sensing signaling proteins, integrin based signals, specific ionic signals like that of  $Ca^{2+}$ ,  $Na^{+}$ , and  $K^{+}$  and electrophoretic movement of intracellular signaling molecules like serotonin. These signals then impinge on gene expression changes For example SLUG/SOX10, PTEN, SIK, NF- $\kappa$ B, Notch. Importantly, these gene expression changes also include ion channel expression that results in establishment of strong feedback loops between the bioelectrical signals and their subsequent gene expression changes. These transcription and translation signals can regulate cell behavior like mitosis, fate determination, differentiation, migration, etc. Thus bioelectrical signals control cell maintenance, cell number, cell fate, and differentiation during physiological processes by employing the canonical biochemical and genetic pathways.

system (Li et al., 2002) provides a plethora of testable hypothesis. No known voltage-regulated role for voltage sensitive phosphatase has been elucidated during patterning *in vivo*. Interestingly however, phosphatase PTEN is an integral regulator of stem cells. In planaria, knock-down of PTEN results in hyperproliferation of stem cells and significantly impaired differentiation capacity (Oviedo et al., 2008b). Similar key regulatory roles of PTEN are demonstrated in hESC and mouse embryonic and adult neural stem cell's growth and differentiation (Alva et al., 2011; Qu and Shi, 2009). A direct interaction between electrical signals and PTEN has also been shown (Zhao et al., 2006): inhibition of PTEN enhances electric field-guided cell migration and wound healing, while inhibition of PI(3)K inhibits the effect of electric signals. Given the importance of phosphatase and bioelectric signals in regulating stem cell maintenance and differentiation, stem cells are a likely context in which to search for a functional role of voltage sensitive phosphatase as transducer of  $V_{\text{mem}}$  change. Modulation of PTEN signaling by the voltage-dependent activity of phosphatases (Zhao et al., 2006) would provide an extremely rich toolkit for physiological (non-genetic) manipulation of intracellular signaling and genetic control of stem cell maintenance and differentiation. These elements can be capitalized upon for the design of bioelectrical intervention in regenerative processes.

A very recent discovery in terms of long-distance cell-cell communication are the tunneling nanotubes (Chinnery et al., 2008; Onfelt et al., 2004; Rustom et al., 2004). These are F-actin membrane tubes up to 200  $\mu\text{m}$  in diameter spanning several cell diameters in length. Tunneling nanotubes facilitate intercellular exchange of small molecules and organelles. Interestingly, a bidirectional  $V_{\text{mem}}$  sensitive bioelectric signal conductance through these tunneling nanotubes has been documented (Wang et al., 2010). GJC were found at both ends of nanotubes and were necessary for bioelectric signal as blocking GJC inhibits electrical coupling of the cells. *In vivo* migrating neural crest cells have been shown to be coupled by tunneling nanotubes (Teddy and Kulesa, 2004) and thus might play an important role in coordinating their migration and differentiation.

Overall, the mechanism of bioelectrical signal transduction has just begun to be understood. Although some mechanisms have been discovered over the past decade, much remains to be explored in this rich area of investigation. Fully capitalizing on bioelectric cues for the control of stem cell behavior will require identifying not only the upstream factors regulating ion channel and pump expression and function in stem cells, but also tracing the specific transduction mechanisms that each type of cell uses to convert signals mediated by  $V_{\text{mem}}$  change into transcriptional responses.

## BIOELECTRICAL CUES MEDIATE HOST CONTROL OF PATTERNING

### Stem Cell Mediated Regeneration as an Information-processing Problem

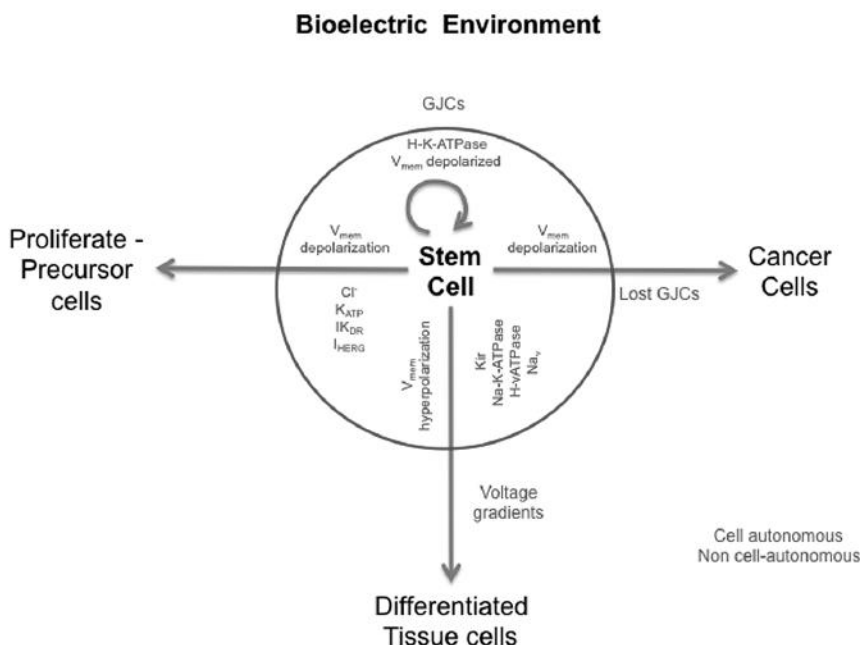
All cells in the body are immersed in physical, chemical, and electrical cues that make up a rich field of information. These signals provide information about a cell's position within the host and enable individual cell behaviors to be exquisitely orchestrated into the systems-level properties of organism pattern, including large-scale shape, orientation of organs, size control, anatomical polarity, etc. (Levin, 2011; Levin, 2012). Understanding and learning how to manipulate the morphology resulting from stem cell behavior is essential, if stem cell biology is to fulfill its full potential for regenerative medicine. Teratomas are tumors that form when embryonic stem cells differentiate into tissues but fail to establish large-scale 3D organization. Thus, beyond specifying individual cell types during stem cell differentiation, it is crucial to learn to coordinate their activity in producing the required biological pattern; bioelectrical signals are a powerful and now tractable set of control knobs for this purpose.

### Bioelectric Cues in Stem Cell-driven Patterning

Ion-based physiological processes guide stem cell activity during complex pattern formation. Functional experiments have shown that some bioelectric events provide specific instructive signals regulating embryonic cell behavior during development and regenerative repair (Borgens, 1984; Jaffe and Nuccitelli, 1977).  $V_{\text{mem}}$ , voltage gradients (electric fields) through tissue and surrounding fluids, iso-electric and iso-pH cell groups established by gap junctions (Fitzharris and Baltz, 2006), and fluxes of individual ions, all carry information to the stem cell as well as to its neighbors, and in some cases, to distant locations (Figure 3). Roles for endogenous currents and fields were found in numerous systems, and in several cases, spatially instructive signaling was demonstrated (Dimmitt and Marsh, 1952; Marsh and Beams, 1947, 1952; Rose, 1972).

Endogenous bioelectric and biochemical cues together co-ordinate stem cell behavior and pattern formation *in vivo*. In frogs, a small and sparse population of embryonic neural crest cells that expressed the glycine receptor  $\text{Cl}^-$  channel were depolarized by manipulation of its activity (Blackiston et al., 2011). This resulted in a large embryonic stem cell population of neural crest that gives rise to melanocytes that overproliferate and acquire a neoplastic phenotype with inappropriate migration and colonization of numerous tissues (Blackiston et al., 2011; Morokuma et al.,





**Figure 3. Bioelectric environment governing the direction of stem cell fate.** Bioelectric signals help control which of the four main paths any stem cell takes: stay quiescent, proliferate and give rise to progenitor or precursor cells, differentiate into specific cell type, or generate a neoplastic cancer cell population. Cell-autonomous events (changes of transcription or gating of the stem cells channels and pumps) are largely encoded by changes in  $V_{mem}$  and control proliferation/differentiation decisions. The non-cell autonomous signals like external electric fields generated by epithelia and GJC communication to nearby cells serve to coordinate stem cell activity with other cell populations in the host providing cues needed for control of cell number and type (such as positional information cues). Misregulation of either set of cues may be one source of cancer, in which normal proliferation and activity of stem cells is driven towards growth that lacks large-scale patterning information (tumors).

*Color image of this figure appears in the color plate section at the end of the book.*

2008). The  $V_{mem}$  signal was conveyed to the non-glycine receptor  $Cl^-$  channel neural crest stem cells via extracellular serotonin. Similar depolarization-mediated induction of neoplastic phenotype was also observed in human melanocyte cultures (Blackiston et al., 2011). Thus, bioelectric signals can divert normal functions of stem cell progeny *in vivo* and are an important factor for preventing, detecting, and perhaps normalizing some types of birth defects and cancer.

Planaria have incredible regenerative capacity due to the presence of high numbers of stem cells (neoblasts) (Oviedo et al., 2003; Reddien and Sanchez Alvarado, 2004). Bioelectric cues have been shown to be crucial for injury induced regenerative patterning from these stem cells (Beane et al., 2011). Depolarization by the activity of the  $H^+K^+$ -ATPase ion exchanger

**Table 2.** Non-mammalian stem cells the bioelectric signals regulating their specific paths.

| Species  | Cell type                             | Channel /current expressed        | Bioelectric property      | Phenotype                                     | Modification of property                     | Effect                 | Phenotype                                   | References                                       |
|----------|---------------------------------------|-----------------------------------|---------------------------|-----------------------------------------------|----------------------------------------------|------------------------|---------------------------------------------|--------------------------------------------------|
| Xenopus  | Embryo                                | KCNQ1                             |                           |                                               | Suppress KCNQ1 by mink                       | Depolarize             | Neoplastic melanocytes                      | (Morokuma et al., 2008)                          |
| Xenopus  | Neural Crest cells                    | $I_{HERG}$ and $IK_{DR}$          | Depolarized $V_{mem}$     | Proliferation                                 |                                              |                        |                                             | (Arcangeli et al., 1997)                         |
| Xenopus  | Neural Crest cells                    | $I_{HERG}$ , $IK_{DR}$ , $I_{Na}$ | Hyper-polarized $V_{mem}$ | Differentiation                               |                                              |                        |                                             | (Arcangeli et al., 1997)                         |
| Xenopus  | Lateral neural crest instructor cells | GlyCl – $Cl^-$ channel            | Hyper-polarized           | Non-cell autonomous regulation of melanocytes | Activate GlyCl or other depolarizing channel | Depolarize             | Overproliferative and neoplastic melanocyte | (Blackiston et al., 2011; Morokuma et al., 2008) |
| Xenopus  | Neural                                | Na-K-ATPase<br>V-ATPase<br>$Na_v$ | Hyper-polarize            | Differentiation                               |                                              |                        |                                             | (Messenger and Warner, 1979)                     |
| Planaria | Neoblasts                             | H-K-ATPase                        | Depolarize                | Regeneration of nervous tissue and head       | Inhibition of H-K-ATPase                     | Prevent depolarization | Prevent regeneration                        | (Beane et al., 2011b)                            |

is crucial for stem cell-mediated regenerative patterning of the nervous tissue (brain and eyes) and the head as a whole. Inhibition of  $H^+K^+$ -ATPase activity induced the formation of a non-head structure at an anterior-facing wound blastema, whereas depolarization via  $H^+K^+$ -ATPase independent means rescued regeneration. Similar observation has been made in the mouse cortex, where the polarizing activity of the V-ATPase  $H^+$  pump is essential for maintenance and differentiation of embryonic NSC (Lange et al., 2011). These data clearly demonstrate the role of  $V_{mem}$  mediated signals in dictating the anatomical pattern resulting from the activity of stem cells during regeneration.

The neural tube develops to become the brain and spinal cord. A very large voltage difference (up to 1,800 mV/mm) is measured across the neural tube in amphibians (McCaig et al., 2005; Shi and Borgens, 1994). The voltage gradient is largely due to transport of  $Na^+$  ions out of the lumen. This voltage gradient is crucial for proper differentiation and patterning of NSC (Borgens and Shi, 1995). The collapse of this voltage gradient (pharmacologically) causes major abnormalities in embryonic neuroblast differentiation and CNS development. Particularly interestingly is that the collapse of the voltage gradients disaggregates already induced structures without cell death (Borgens and Shi, 1995) resulting in just a mass of cells waiting for differentiation cues. Division and differentiation of NSC begins at the lumen of the neural tube where steepest bioelectric gradients are found. Moreover, the thinnest part of the neural tube (the floor plate) has the largest gradient across it and this provides dorso-ventral spatial patterning cues important for CNS development and neuronal differentiation (Wood and Willits, 2006; Wood and Willits, 2009). These data strongly suggest that bioelectric cues provide important patterning information to embryonic NSC towards CNS specification.

Cell-autonomous and long-range signaling mechanisms, including electrophoretic transport of morphogens (Fukumoto et al., 2005), diffusible secondary messengers (Blackiston et al., 2011), GJC (Esser et al., 2006), and tunneling nanotubes (Wang et al., 2010), allow bioelectrical gradients to carry information in several different modes. For example,  $V_{mem}$  levels specify tissue and organ identity in stem cells, as seen in the ability of a specific  $V_{mem}$  range to drive entire cascade of eye formation and induce complete ectopic eyes (including organized retinal neurons) in embryonic cells at locations far away from the anterior neuroectoderm that is normally competent to make eye (Pai et al., 2012). These data underscore the importance of bioelectrical gradients in determining stem cell mediated tissue/organ specification. Changes in  $V_{mem}$  and ion content may provide master-regulator-like triggers that initiate complete, highly orchestrated, and self limiting downstream patterning cascades to generate tissues from stem cells. Molecular and cell biology are now being applied to understanding the role of specific ion

transporter activity in control of adult stem cells and regenerative potential, and the switch between stem cell and neoplasia (Adams and Levin, 2006; Levin et al., 2002; Morokuma et al., 2008; Nogi and Levin, 2005; Oviedo and Levin, 2007b; Sundelacruz et al., 2008).

## **Mechanism of Modeling Stem Cell Patterning Information Coded by Bioelectric Signals**

Currently, we lack a full understanding of the bioelectric signals that induce stem cell-mediated development and regeneration of tissues/organs to proper shape with respect to the target pattern of the organism. Because the activity of ion channels, pumps, and gap junctions are controlled post-translationally, the bioelectric signals they give rise to are largely undetectable by popular proteomic and mRNA profiling strategies: cells with exactly the same complement of ion translocator mRNAs and proteins can be in very different physiological states. Hence, comprehensive physiomic techniques are needed to profile bioelectrical signals. There is a lack of repository database of studies and experiments on bioelectric signals, linking physiological parameters to stem cell fate determination and the encoded target morphology. True control over stem cells in pattern formation will necessitate use of such repository database information to develop *in silico* predictive models showing how each signal guides stem cell fate decisions. This is required to help scientists and clinicians decide what signals to provide and when, to achieve the desired stem cell fate and organ patterning *in vivo*.

The mapping between bioelectrical signals and the respective stem cell outcomes remain to be discovered and compiled into databases that can be quantitatively mined. One model predicts that stem cells similar in profile (in terms of differentiation potential and plasticity) will cluster together in a multi-dimensional state space where each axis is defined by particular bioelectric, biochemical, and genetic parameter. Using genetic or pharmacological approaches, the cells could then be moved from their current state into a desired state (of differentiation). Such a model would go far towards a predictive control of stem cell behavior. However, this requires gathering of tremendous amount of physiomic profiling data of different stem cells, their differentiation states and target tissues in various model systems (invertebrates like planaria to amphibians like xenopus and axolotl to mammals like rodents and ultimately humans). These would then have to be integrated into the current genetic profiling and developmental database algorithms. The rewards of such intensive profiling and database generating tasks would be enormous for developmental, biomedical, and regenerative medicine.

## BIOELECTRICITY AND CANCER STEM CELLS

Many cancers are clonal in origin and have been suggested to mainly develop from transformation of resident adult stem cells giving rise to cancer stem cells (Reya et al., 2001). Many commonalities have been found between adult stem cells and cancer stem cells, including endless proliferative potential, capacity to give rise to heterogeneous population of cells etc. Using this logic, and ignoring the role of instructive signals from the environment, one would make the prediction that higher regenerative capacity of an organism (access to stem-like cells) would lead to higher susceptibility to cancer. However, stem cell regeneration and cancer have a fascinating inverse relationship (Brookes, 1998; Pizzarello and Wolsky, 1966; Tsonis, 1987). Species such as newt and axolotl have remarkable regenerative capacity in certain tissues like tail, limbs, lens, and craniofacial tissues including some parts of brain and spinal cord (Brookes, 1998). Interestingly, these regenerating tissues also show high resistance to carcinogenesis (Del Rio-Tsonis et al., 1992; Donaldson and Mason, 1975). The regeneration process involves generation of stem-like cells (in the blastema) by de-differentiation or trans-differentiation upon injury or amputation (Ferretti and Brookes, 1988; Rieder and Hard, 1990). The fascinating observation is that exposure of these blastema stem-like cells to carcinogenic chemical and UV light results in induction of an extra organs limb or lens instead of a tumor (Breedis, 1952; Butler and Blum, 1955; Eguchi and Watanabe, 1973). Similarly, if limb regeneration is initiated by cutting in the middle of a tumor, the tumor cells can be transformed into normal limb tissue (Rose and Wallingford, 1948). These data suggest that patterning signals during regeneration are able to control cell behavior even of genetically transformed cells.

Endogenous bioelectric signals provide important patterning information during development and regeneration. Involvement of bioelectric cues in normal patterning versus carcinogenesis is demonstrated in a series of experiments in frogs (Blackiston et al., 2011). In the embryonic stem cell population of neural crest cells there is a small subset of “instructor cells” (expressing glycine  $\text{Cl}^-$  channel) that governs the generation and migration of melanocytes from the neural crest cells. Depolarizing the  $V_{\text{mem}}$  of these instructor cells by manipulating the glycine  $\text{Cl}^-$  channel activity induces neoplastic characteristics in the melanocytes that overproliferate and migrate to colonize numerous tissues (Blackiston et al., 2011). Similar effect of depolarization mediated neoplastic like morphology was also observed in human melanocyte cultures (Blackiston et al., 2011). These set of data indicate that melanocytes, which are stem cell derivatives become cancerous not by genetic transformation of stem cells, but rather by change in the patterning signals.

Alterations in  $V_{\text{mem}}$  and ion channel expression/function have been observed in a wide array of cancers (Kunzelmann, 2005). Cancer cells have been observed to be depolarized with respect to normal healthy tissue (Arcangeli et al., 1995; Binggeli and Weinstein, 1986; Pardo, 2004). A major difference between stem cells and cancer cells is the GJCs. Stem cells are coupled by GJC which creates a uniform bioelectric informational niche. Loss of GJC accompanies early steps in neoplastic transformation (Omori and Yamasaki, 1998; Pitts et al., 1988). Conversely, neoplastic characteristics can be suppressed by ectopic induction of GJC in tumor tissue (Hellmann et al., 1999; Mehta et al., 1991; Rose et al., 1993). This suggests a role of GJC in mediating the bioelectric information flow that is necessary for maintaining stem cells niche, which is lost in tumors (Pierce, 1983; Pierce et al., 1986; Rubin, 1985; Ruiz i Altaba et al., 2004). Hence, manipulation of bioelectric patterning cues could be used as plausible control points for cancer stem-cell normalization.

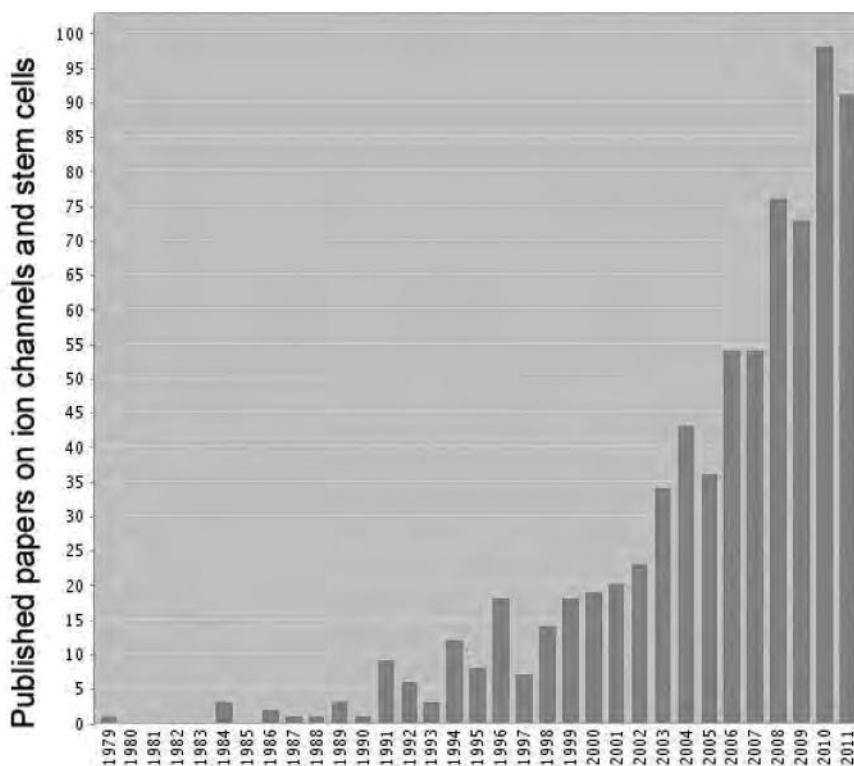
These set of data strongly support a patterning control of stem cells behavior (transformed and normal). Normalization or rebooting strategies (Ingber, 2008) that reconnect the cancer stem cells to the normal patterning signals of the host (Rose and Wallingford, 1948; Trosko et al., 2004; Yamasaki et al., 1995) like those found during embryonic environments are known to reverse the cancer phenotype (Hendrix et al., 2007; Mintz and Illmensee, 1975). This strongly suggests tumors as manifestation of aberrant stem cell instructive information. Since bioelectric signals serve as a prominent patterning information carriers, they can now be used in such cancer normalizing strategies. Initial steps towards bioelectric control of cancers has already being successfully conducted (Kirson et al., 2007; Kirson et al., 2009a; Kirson et al., 2009b; Stupp et al., 2010).

## CONCLUSIONS AND FUTURE PERSPECTIVES

We have outlined exciting recent data implicating bioelectric signals such as resting potential ( $V_{\text{mem}}$ ) in regulation of stem cell behavior (Figure 3). Although bioelectric signals' involvement during embryonic development and wound repair has been documented for almost a century, recent technological advances in molecular physiology techniques have led to a reinvigoration of this important field. While most of the data have been derived in differentiated tissues, studies increasingly implicate ion transporters and bioelectric parameters in stem cell biology. It is now clear that a variety of embryonic and adult neural and mesenchymal stem cell types are sensitive to internal and external endogenous bioelectrical events, and such ion-based signals are an important part of the stem cell niche communication. At the same time, many of the same questions are open for stem cells as for somatic cells. These include for example the

quantitative nature of the bioelectric code (mapping between voltage profile and differentiation trajectory) and the importance of subcellular gradients (e.g., nuclear envelope potential).

Further development of even higher-resolution tools (optogenetics, better voltage dyes, etc.) will greatly advance this aspect of stem cell biology, revealing not only convenient “control knobs” for manipulation of stem cell behavior in biomedical or bioengineering contexts, but also identifying fascinating new pathways by means of which stem cell activity is coordinated into the system-level anatomical needs of the host organism. An analysis of published work in the field (Figure 4) shows that ion channels are increasingly being implicated in studies of stem cells. Crucially, such work must not only study the genetic pathways (specific channels and pumps and their regulation during the stem cell life cycle) but truly understand the *physiology* of the system, since ion translocators and gap junctional



**Figure 4. Publications on ion channels and stem cells in the scientific literature.** Data was taken from ISI Web of Knowledge. Over the last 3 decades the number of studies of ion channels in stem cell biology has risen dramatically. In addition to molecular genetics of specific channel genes expressed in stem cells, this work should be integrated into the bigger picture of bioelectrical signals (as distinct from whatever specific gene or protein implements a given physiological state) as regulators of cell behavior within the anatomy of the organism.

connections can result in dynamical spatio-temporal changes of bioelectrical properties (self-organization and feedback behavior) among cell groups without changes of mRNA or protein. Interpreting data on ion channel expression or physiological parameters (voltage signatures of stem cell subpopulations for example) should be done in the context of bioelectricity as a patterning signal broadly relevant to morphogenesis. Understanding and learning to control such physiological networks, which are largely invisible to modern profiling techniques focused on biochemical signals and transcriptional events, will be a crucial aspect of fully capitalizing on the promise of stem cell biology. This is likely to have transformative impact not only on regenerative medicine and developmental biology, but also on synthetic and cancer biology.

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## ABBREVIATIONS

|       |                                                     |
|-------|-----------------------------------------------------|
| SC-CM | stem cell-derived cardiomyocytes                    |
| GJC   | gap junctional channels                             |
| Kcnq  | potassium voltage-gated channel, KQT-like subfamily |
| Kcne  | potassium voltage-gated channel, Isk-related family |
| Snai  | snail homolog                                       |
| Sox   | SRY-box containing gene                             |

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## **PART II**

# **TISSUE FORMATION DURING DEVELOPMENT**

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# Development from the Fertilized Egg to the Three Germ Layers and its Relevance to Signaling and Lineage Commitment of Embryonic Stem Cells

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## SUMMARY

In this chapter we cover developmental biology of murine development from the fertilized egg to lineage commitment and specification of the three germ layers. Post-fertilization processes including pluripotency, pre- and post-implantation development and axis specification are discussed in detail. We go on to discuss the role of the Tgf- $\beta$ , Nodal, Activin, Bmp, Fgf and

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*List of abbreviations after the text.*



Wnt signaling pathways in axis formation and gastrulation and the roles of the various early embryonic lineages and signaling centers in the formation of the primitive streak. Post-primitive streak cell lineage allocation and the final specification of ectoderm, mesoderm and endoderm are presented. We conclude with a comparison of the similarities and differences between early murine developmental biology and mouse embryonic stem cell (ESC) differentiation as embryoid bodies (EBs) which share a number of features with embryo development. Finally we discuss the differences and relevance of the mouse model to human embryonic stem cell (hESC) differentiation and the relationship between early embryonic subtypes such as the inner cells mass (ICM) and epiblast (EPI) with reprogrammed induced pluripotent stem cells (iPSC) and epi-iPSC.

## INTRODUCTION

Stem cells retain the ability to undergo cell division while maintaining their differentiation potential through a process known as self-renewal. In contrast to adult stem cells, pluripotent stem cells, consisting of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can produce all cell types of an embryo proper (Okita and Yamanaka, 2010; Wobus and Boheler, 2005). In fact, an entire ESC derived embryo, excluding some extra-embryonic tissues, can be formed from mouse ESCs through a process known as tetraploid aggregation or via chimera formation following injection into a blastocyst. Importantly, ESCs from mouse and human can be isolated from early embryos and indefinitely maintained in culture. These self-renewal properties with unlimited cultivation and pluripotent potential for differentiation have led to rapid advances in our understanding of developmental biology.

The developmental potential of mammalian ESCs is not equivalent *in vivo* and *in vitro*. ESCs can only form an intact and viable organism when placed in an appropriate *in vivo* environment (e.g., blastocyst) where appropriate spatial cues, growth factor gradients and signals are maintained. While many of the same signaling mechanisms are conserved *in vitro*, the absence of morphogenesis, spatial cues and appropriate gradients preclude the formation of a viable organism. Under these *in vitro* conditions, ESC development is chaotic. Moreover, gaps in our understanding of mouse and human development make it difficult to adequately mimic development *in vitro*. In part, this is due to the size of early mammalian embryos and ethical concerns surrounding the use of human embryos. Post-implantation, developing embryos also establish physical connections with the mother rendering the embryos difficult to access. It is currently impossible to imitate maternal interactions *in vitro* to study developmental changes. Despite the lack of appropriate morphological and spatial cues, ESCs can and do form

all cells from three primary germ layers *in vitro*; however, many *in vitro*-derived ESC progenies are embryonic in nature and functionally immature. ESC development outside of a native context is, therefore, best described as a model of *in vitro* differentiation.

An in-depth knowledge of early development and of the process of gastrulation is required to differentiate ESCs into viable and functional cells of one of the primary germ layers while accurately mimicking *in vivo* ontology and lineage formation. Although limited information on germ layer induction and the signaling pathways involved in human gastrulation are available, the processes involved in early mouse development and gastrulation are well understood (Tam and Loebel, 2007). In this chapter, we review early mammalian development using mouse as the primary source of information from pre-implantation through early gastrulation. We describe signaling events involved in lineage formation and activation of transcriptional regulators, and how the factors interact to generate cells of the primary three germ layers. Some comparisons are also made between development and known properties of ESC potency and early differentiation. These relationships are meant to explain the biology of ESCs, highlight their limitations relative to normal development, and foster an understanding of the potency of ESCs, signaling events, and lineage commitments through early gastrulation that occur with differentiation.

## Embryonic Development

To correctly describe mammalian development and to contrast it with *in vitro* differentiation, several terms should be defined.

First, cell lineages or cell fates of an organism are accurately defined as the pattern of cell divisions and cell types that occur during development. In some organisms like *C. elegans*, cells display invariant patterns of cell lineage formation. In other organisms, including mammals, lineage patterns during development are variable and are not always highly correlated with cell fates; but in conjunction with other signaling mechanisms, these processes foster cell specification, commitment, and terminal fate decisions. Similarly, ESCs typically demonstrate variable cell fates in culture; however, these choices are stochastic in nature. This is due, in part, to a variety of environmental factors without spatial controls that are affected by variable culture conditions (cell density, media, oxygen), growth factor concentrations, extracellular molecule signaling, and (dys)regulated gene expression patterns.

Second, cell specification or commitment can be divided into two categories: autonomous and conditional specification. The former is a self-directed property that depends on all cytoplasmic components present in the cell, including RNAs and proteins. In some instances, the cytoplasmic

components are not always equally divided between daughter cells during division, which results in asymmetric cell properties and possibly distinct fate choices. Conditional specification is a cell-extrinsic process that relies on cues and interactions between cells or from concentration-gradients of morphogens. Thus specification refers to a cell's plasticity and ability to make fate choices depending on the internal or external environment.

Third, determination refers to a state where a cell's fate cannot be altered, at least not under normal conditions. Moreover, determination is usually followed by differentiation, where actual changes in structural or functional properties result in specific cell types.

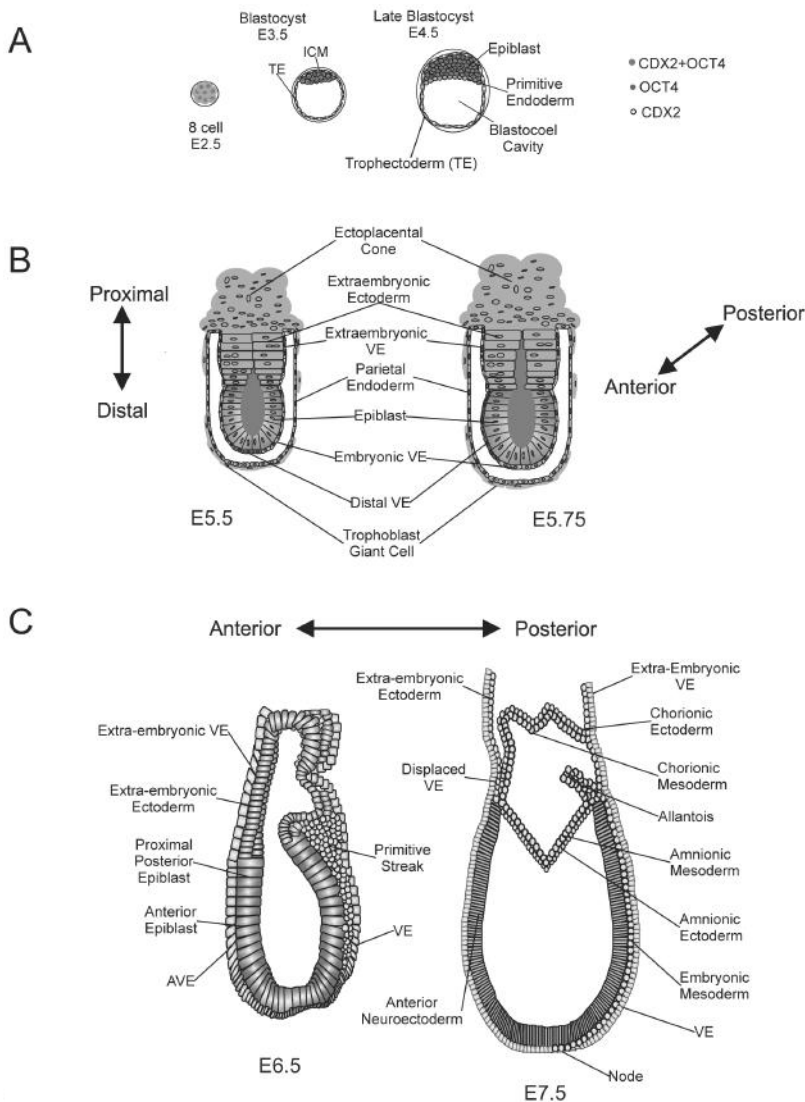
Finally, three types of cell division contribute to specification and fate choices. The first type is symmetric where a cell divides into two daughter cells identical to the parental cell. In the case of stem cells, this form of division generates two identical stem cells. The second and third types are asymmetric. In one, the parental cell produces one identical cell (self-renewal) and one cell that differs from the parental cell (differentiated progeny). The other is diversifying cell division, where two unique differentiated cell types are produced from one parental cell. Both of these forms of asymmetric cell division represent a form of specification that occurs during early embryo development.

## **Pre-implantation Development**

Development is initiated in mammals when a sperm fertilizes a viable oocyte. After fertilization, the metaphase II arrested oocyte nucleus completes meiosis, and the two parental pronuclei in the zygote fuse to become diploid. In most species, the initial phase of development depends largely on maternal transcription products (RNA and protein) present in the fertilized embryo. In mice, transcriptional control of development passes from maternal gene products to those transcribed from the newly formed embryo before the 2-cell stage. This transition is accompanied by rapid maternal transcript degradation; however, maternally encoded proteins may remain functional for much longer periods of time. Shortly after fertilization progressive DNA demethylation and epigenetic reprogramming of both the maternal and paternal genomes also begins, excluding genomic imprints. In other mammals, including humans, the transition to embryo genome transcriptional control occurs within one or two subsequent cell divisions. During these earliest symmetric cell divisions, daughter cells are totipotent, and can give rise to all embryonic and extra-embryonic tissues. The potency of these cells becomes more limited with subsequent asymmetric cell divisions (Cockburn and Rossant, 2010).

After the first 2–3 rounds of cell division, mouse blastomeres (cells produced through cell division in the zygote without a change in the overall size of the embryo) show no overt morphological differences. Early blastomeres are totipotent and can give rise to all extra-embryonic and embryonic tissues. At the 8-cell stage in mouse and 8- to 16-cell stage in human, embryos undergo structural changes with an increase in intercellular adhesion in a process known as compaction. This process results in more limited cell potency and the formation of a compact smooth structure known as a morula. Subsequently, all blastomeres flatten and become polarized, leading to a clear distinction between intercellular boundaries and the specification of cell types with restricted cell fates. During the morula stage, cell divisions generate two visibly distinct cell lineages. Initially, octamere-binding transcription factor 4 (*Oct4*) [correctly known as POU domain, class 5, transcription factor 1 (*Pou5f1*)] and caudal-type homeobox protein 2 (*Cdx2*) are co-expressed in all cells present in the compacted morula. Subsequent asymmetric cell divisions result in segregation of cells on the outer and inner surface of the developing embryo. Blastomeres that compose the outer layer of the embryo will differentiate to form the trophectoderm (TE), while the remaining cells form the bipotent inner cell mass (ICM). At the 32-cell stage (E3.5) in mouse, a blastocyst containing a fluid-filled blastocoelic cavity (blastocoele) develops that is essential for the later stages of gastrulation to proceed. At around E3.75, ICM cells become fully committed to either epiblast (EPI) or primitive endoderm (PE, the equivalent of hypoblast in human), but they are only morphologically distinct and fully restricted to their respective cell fates by E4.5 (late blastocyst) (Figure 1).

The earliest fate choice between TE and ICM occurs when *Cdx2*, a transcription factor that suppresses *Oct4*, becomes localized on the surface of the blastocyst. This down-regulation of *Oct4* results in TE specification and the generation of cells with epithelial characteristics (Strumpf et al., 2005). Other factors like *Tead4*, a member of the TEA domain/transcription enhancer factor family and the T-box transcription factor *Eomesodermin* (*Eomes*) are important for TE differentiation. Fibroblast growth factor (*Fgf*) signaling is also critical to the establishment of TE and PE. *Fgf4* is expressed primarily in the ICM, whereas *Fgfr2* is expressed primarily in the trophectoderm, suggesting that the cytokine *Fgf4* acts as a paracrine maintenance factor for the TE *in vivo*. Both *Fgf4*<sup>-/-</sup> and *Fgfr2*<sup>-/-</sup> mouse embryos display post-implantation lethal phenotypes due to TE defects, which further suggest that *Fgf4* acts through *Fgfr2* in the early mouse embryo and that this signaling is essential for the maintenance of the first cell lineage specified in the mouse embryo—the TE (Arnold and Robertson, 2009; Cockburn and Rossant, 2010).



**Figure 1.** (A) Pre-implantation embryos, showing the 8 cell-staged embryo, and early and late stage blastocysts. In the early embryo, Oct4 and Cdx2 are co-expressed in all cells. E3.5–E4.5 blastocysts have Cdx2 and Oct4 partitioned to the TE and EPI. (B) E5.5, ICM (blue cells) and primitive endoderm formation (red cells), visceral endoderm, extra-embryonic ectoderm (black cells) and epiblast formation (blue cells). E5.75, movement of the visceral endoderm. (C) E6.5, primitive streak formation and E7.5, continued development of the primitive streak and embryonic and extra-embryonic mesoderm formation. ICM, inner cells mass; VE, visceral endoderm; AVE, anterior visceral endoderm. Diagram adapted from Lu et al. (2001).

Color image of this figure appears in the color plate section at the end of the book.

Just as a unique set of active genes and signals are required for TE specification, other gene sets are required for fate determination of ICM cells to PE and EPI. These include genes encoding *Oct4*, *Sry* (sex determining region Y)-box 2 (*Sox2*), the homeodomain-containing transcription factor *Nanog*, Spalt transcription factor Sal-like4 (*Sall4*), and Gata-binding factor 6 (*Gata6*). Together with *Sox2*, *Oct4* suppresses *Cdx2* expression and up-regulates other developmental genes (e.g., *Fgf4*) associated with EPI. *Nanog* suppresses PE formation, while *Sall4* is required for the development of both EPI and PE from ICM. In contrast, the transcription factor *Gata6* is highly expressed in PE and effectively drives endoderm formation. *Fgf* signaling may also be crucial for the segregation of PE from EPI lineages through its regulation of *Gata6* gene activity.

ICM commitment depends on the wave of asymmetric cell division that occurs in the early embryo. Cell fate studies (Morris et al., 2010) have shown that symmetric divisions at E2.5 (8- to 16-cell stage) generate primarily EPI, while subsequent divisions at E3.5 (16- to 32 and 32- to 64 cell stages) generate PE. Initially, these cells display some degree of plasticity (the cells can become either EPI or PE) and express high levels of both EPI and PE markers like *Nanog* and *Gata6*. Due to positional induction mechanisms and adherence differences, PE committed cells with high levels of *Gata6* and low levels of *Nanog* segregate to the surface of the ICM to contact the blastocoelic cavity, polarize, and express components of the basal lamina. Committed EPI cells that do not contact the blastocoel express high levels of *Oct4* and *Nanog*. Moreover, EPI cells, from which ESCs are derived, have been described as the “true ground state” since these cells give rise to all somatic and germ cells of the embryo proper, as well as to extra-embryonic mesoderm.

Thus the late blastocyst contains three distinct lineage-restricted populations of cells. The TE controls implantation and gives rise to progenitors of the placenta, including the extra-embryonic ectoderm and the ectoplacental cone. The PE generates parietal endoderm and the visceral endoderm (VE). The former migrates from the surface of the ICM to directly contact the maternal tissues, while the latter remains in contact with the embryo and expands along the surface of the epiblast and the extra-embryonic ectoderm. EPI gives rise to extraembryonic mesoderm and the embryo proper, including all somatic cells and germ cells. The three extra-embryonic endodermal cell layers provide the first contact between the conceptus and the uterus, nurture the remaining central primitive ectoderm, and deliver signals preparing the EPI for gastrulation.

## Axis Specification and Post-implantation Development

At E4.5 the developing embryo reaches the uterus and the blastocyst “hatches” from the zona pellucida and is ready to implant. Between E5.0 and E6.5, the radially symmetric embryo develops an inverted cup-shaped epiblast that is surrounded by visceral endoderm. For this cup-shape to develop, a cavity forms in the center of the epiblast and elongates along the proximal-distal (P-D) axis to form an embryo at the “egg-cylinder” stage, where the extra-embryonic ectoderm (ExE) layer of epithelial cells is located proximally and juxtaposed to cells of the EPI. Both cell types are covered by a continuous monolayer of visceral endoderm (VE). The parietal endoderm and trophoblast loosely surround the entire embryo, and the ectoplacental cone, a trophoctoderm derivative, is located on the proximal-most edge of the conceptus.

The establishment of axis specification in the embryo can be considered the starting point of pattern formation, which is critical for subsequent cell lineage distribution and tissue development. Both the ExE and EPI surround the proamniotic cavity (Tam and Behringer, 1997), and signaling among EPI, visceral endoderm and extra-embryonic ectoderm through the secretion of morphogens leads to regionalized gene expression patterns and the first signs of tissue regionalization along the P-D axis of the developing embryo. The primary signaling molecules involved in these processes include members of the Tgf- $\beta$  (Nodal, bone morphogenetic protein (Bmp)), Wnt, and Fgf families (Arnold and Robertson, 2009).

At E6.0, anterior-posterior (A-P) patterning of the embryo begins (Perea-Gomez et al., 2004). A fundamental process during the generation of the A-P axis is the formation of the primitive streak. In fact, the appearance of the primitive streak is the first symmetry-breaking event in the embryo and marks the beginning of gastrulation. In mice, the primitive streak is localized to a region of the EPI located next to the extra-embryonic ectoderm where it marks the posterior site of the embryo. At E6.5, gastrulation commences after cleavage and the formation of the blastula, and on the posterior side of the embryo, the primitive streak. Effectively, the primitive streak serves as a conduit for the generation of mesoderm and definitive endoderm. During gastrulation, some epiblast cells undergo an epithelial to mesenchymal transition, ingress (move with pseudopods) via morphogenetic cell movements and migrate along the outer surface towards the anterior pole to form mesoderm and endoderm. At E7.5 mesodermal cell types become patterned to their respective fates and endoderm and the node are formed. These two tissues and the ectoderm, the epiblast descendants that do *not* pass through the primitive streak, constitute the three primary germ layers that contain progenitors of all embryonic tissues as well as the extra-embryonic mesoderm of the yolk sac, the allantois, and the amnion. Each

primary germ layer subsequently gives rise to specific tissues and organs. The ectoderm produces epidermis, the neural crest, and other tissues that will form the nervous tissue. The endoderm contributes to the epithelium of the digestive and respiratory systems and associated organs like liver and pancreas. The mesoderm, which morphologically is located between the ectoderm and endoderm, generates muscle, cartilage, bone and connective tissue, dermis, notochord, blood and blood vessels, and organs like heart.

## **Signaling Pathways in Development**

Several signaling pathways are critically involved in the process of axis specification during development, including members of the Tgf- $\beta$ , Wnt, and Fgf family members. A brief overview of their generalized functions is presented in this section. Other signaling pathways such as those for Notch or Hedgehog signaling are also very important, but these will be only briefly discussed.

The Tgf- $\beta$  superfamily consists principally of Tgf- $\beta$ /activin, Bmp/growth-differentiation factors (Gdf) and Nodal. These family members transmit signals from the membrane to the nucleus primarily through binding to a serine/threonine kinase heteromeric complex consisting of Tgf- $\beta$  type I (Tgfbri) and type II (Tgfbrii) receptors. The type I receptor, which is also known as activin receptor-like kinase, (Alk) acts downstream of the type II receptor and propagates signals through Smad (small mother against decapentaplegic) molecules that function as intracellular signal transducers (Massague and Wotton, 2000). The eight known Smad proteins can be classified into three functional classes: (i) receptor-regulated Smads (Smad1, 2, 3, 5, and 8), (ii) co-mediator Smad (Smad 4), and (iii) inhibitory Smads (Smad6 and 7). Tgf- $\beta$  and Nodal signaling activate primarily Smad 2 and 3 phosphorylation through Alk-4, -5, and -7, while Smads 1, 5 and 8 are phosphorylated by Bmp activation of Alks-1, -2, -3, and -6 (Massague and Wotton, 2000; Wang and Tsang, 2007). In its phosphorylated form, receptor regulated Smads bind with essential components of the transcriptional network to induce gene expression. Importantly, Nodal and activin both trigger activin receptors, but unlike activin, Nodal requires Egf-Cfc co-receptors (i.e., Tdgf1 (Cripto) and Cryptic in mouse and human, respectively) for signaling and transcriptional control. Left-right determination factors 1 (Lefty1), a divergent member of the Tgf- $\beta$  superfamily, also functions as an antagonist of Nodal signaling (Figure 2).

Wnt proteins (or Wg/Wingless) belong to a family of highly conserved secreted signaling molecules that also control cellular processes such as embryonic induction, cell fate specification, differentiation, and the generation of cell polarity. Wnts trigger signaling pathways after binding to a membrane surface protein of the Frizzled family, but the recruitment



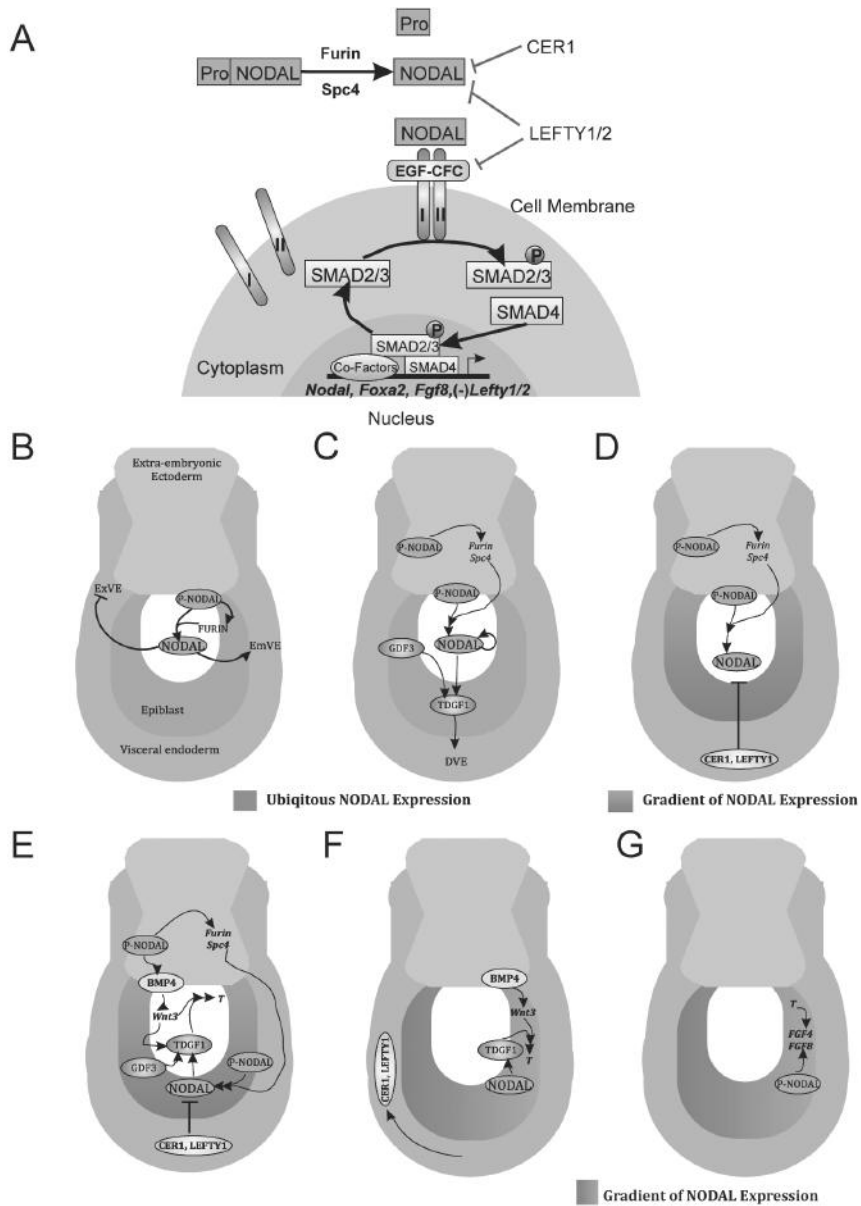


Figure 2. contd....

of proteins in the cytoplasm depends on which Wnt pathway is activated. In the canonical pathway, binding of Wnt proteins to Frizzled causes the receptors to activate Dishevelled (Dsh) family proteins that are a normal component of the receptor complex. Once activated, it acts to inhibit a protein complex containing axin, glycogen synthetase kinase-3 (Gsk-3), and adenomatous polyposis coli (Apc). This latter complex normally promotes degradation of  $\beta$ -catenin, but when DSH is activated,  $\beta$ -catenin stabilizes and can translocate to the nucleus where it exerts its effects through TCF/LEF factors (Logan and Nusse, 2004). In contrast, the non-canonical pathway is PKC-dependent and involves G-proteins, the transmembrane protein strabismus, phospholipase C, c-Jun Kinase (Jnk), Rho family GTPases, and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II. Wnt proteins have been classified into two groups: the Wnt1 group (Wnt1, Wnt3a and Wnt8a) which transduce signals through the canonical pathway (Logan and Nusse, 2004); whereas, the Wnt5a group (Wnt4, Wnt5a and Wnt11) act mostly through the non-canonical pathway (Katoh and Katoh, 2007; Katoh, 2011). The secretion of inhibitors such as Cerberus (Cer1), Dickkopf homologue

**Figure 2.** Nodal Signaling in Development. (A) The Nodal precursors (Pro-Nodal) act as partial agonists when bound to the receptors in a co-receptor independent manner. Pro-Nodal is also cleaved by the convertases Furin or SP4 to generate mature Nodal, which together with its co-receptor (TDGF1) activates the type I-II receptor complexes to phosphorylate the down-stream signalling molecules Smad2/3. Activated Smads associate with co-SMAD to translocate to the nucleus where it regulates gene expression. As a regulatory mechanism, extracellular antagonists (Cer1 and Lefty1 & 2) directly bind to Nodal ligands or receptor complexes to antagonize Nodal signalling. Diagram adapted from Arnold and Robertson, 2009. (B) E5.0, Nodal precursor protein activates the transcription of *Furin* which cleaves the Nodal precursor protein to produce the processed form of Nodal. Nodal signals from the epiblast repress the expression of genes such as *Hnf4a*, *Gata4*, *Ttr* (*transthyretin*) and *Furin* in the extra-embryonic visceral endoderm (ExVE). Simultaneously, in the embryonic visceral endoderm (EmVE) Nodal signals up-regulate the expression of *Lhx1*, *Fgf5*, *Fgf8*, *Bmp2*, *Otx2* and *FoxA2*. (C) E5.25, Nodal precursor protein from the epiblast acts in the extra-embryonic ectoderm to activate the transcription of *Furin* and *Spc4*, by signalling activin receptors 1B and 2A (Acrv1b and Acrv2a). Nodal and Gdf3, working with a co-receptor Tdgf1, specifies the distal visceral endoderm (DVE) (D). E5.5, The graded signalling of processed Nodal from the epiblast/extra-embryonic boundary is additionally antagonised by Lefty1 and Cer1 from the DVE to create the proximal-distal gradient. Diagram adapted from (Tam and Loebel 2007). (E) E6.0, Bmp4 signalling from the extra-embryonic ectoderm activates a regulatory pathway involving Wnt3 which activates *T* expression initially near the epiblast/extra-embryonic ectoderm boundary. (F) E6.25, Movement of the *Cer1* and *Lefty1*-expressing AVE to the anterior side of the embryo establishes an anterior/posterior gradient and the primitive streak forms on the posterior side of the embryo. (G) E6.5, Expression of *Fgf4* and *Fgf8*, which is downstream of *T* and is enhanced by the activity of the Nodal precursor protein, maintains the mesoderm and cells move through the primitive streak. Diagram adapted from Tam et al. (2007).

Color image of this figure appears in the color plate section at the end of the book.

1 (Dkk1) and Crescent (Frzb2) also obstruct Wnt signaling and are critical to its regulation.

In vertebrates, the Fgf receptor (Fgfr) family consists of four genes, *Fgfr1-4*, which undergo alternative splicing in their extracellular domain to generate a wide variety of receptors with different ligand affinities. Signaling is initiated when Fgf ligands bind to the extracellular domain of Fgfr to cause dimerization of the receptors. This leads to the trans-phosphorylation of specific tyrosine residues on the intracellular domain of the receptor tyrosine kinase and binding of signal transducing proteins like PLC $\gamma$ LCdrs2, or Src family members, which triggers activation of the corresponding intracellular pathway (Katoh and Katoh, 2006). During embryogenesis, Fgf signaling plays an important role in the induction/maintenance of mesoderm and neuroectoderm, the control of morphogenetic movements, anterior-posterior (A-P) patterning, somitogenesis, and the development of various organs.

Developmentally, these signaling pathways play critical roles in axis formation, left-right asymmetry, development of the primitive streak, and germ layer specification and determination. Since the roles of Oct4, Sox2, Nanog, and Sall4 have already been discussed; the maintenance of pluripotent EPI cells during development will not be discussed further, but there are several reviews that discuss signaling in pre-implantation development (Arnold and Robertson, 2009).

## **Signaling and Axis Formation During Development**

Polarity of the pre-gastrulation embryo results from reciprocal cell-cell interactions between epiblast and the two extra-embryonic tissues (i.e., extra-embryonic ectoderm (ExE) and visceral endoderm (VE)), and involve Nodal and Fgf signaling. These interactions lead to regionalized gene expression along the P-D axis that subsequently directs A-P tissue identity—the start of embryonic pattern formation. Left-right asymmetry is also established through a process that utilizes Nodal and Lefty proteins. Together, these localized activities establish a “signaling center” or node that is critical to the formation of the primitive streak and mesoderm during gastrulation.

Nodal is first detected in the ICM and PE at E4.5. It is expressed throughout the epiblast at E5.0 and is present in the VE during early post-implantation stages (Varlet et al., 1997). Through implantation and until E5.5, Nodal has a role in maintaining the expression of pluripotency markers Oct4, Nanog, and Foxd3 in the ICM/EPI (Mesnard et al., 2006). With the appearance of extra-embryonic ectoderm (ExE), Nodal activity specifies embryonic VE and restricts the expression of Furin, Gata-binding protein 4 (Gata4), hepatocyte nuclear factor 4a (Hnf4a), and transthyretin (Ttr) to the

extra-embryonic region (Mesnard et al., 2006). Nodal thus acts to promote posterior cell fates in the epiblast and maintain molecular patterns in the adjacent ExE.

Mechanistically, Nodal and the convertase enzyme Furin, which cleaves Pro-Nodal into active Nodal, are co-expressed in PE with a subset of distal visceral endoderm (DVE) markers (e.g., *Lefty1* and hematopoietically expressed homeobox *Hhex*). Pro-Nodal then acts independently of the co-receptor (Egf-Cfc protein) and Smad protein activation in ExE to up-regulate convertase enzyme *Furin* and *Spc4* (*Pcsk6*) genes via binding to Alk2 (*Acvr1*) and to the activin-receptor IIA (*Acvr2a*) (Beck et al., 2002; Ben-Haim et al., 2006). The ExE then secretes these enzymes into the epiblast to cleave Pro-Nodal into mature Nodal (Beck et al., 2002), which amplifies the expression of its co-ligand, *Tdgf1* (Beck et al., 2002; Yan et al., 2002). Nodal-*Tdgf1* signals from the EPI pattern the VE and direct proper orientation of A-P axis movements, which are critical for primitive streak formation. ExE is also the source of bone morphogenetic protein 4 (*Bmp4*).

Nodal signals at E5.0 induce early P-D patterning in the EPI by activating Smad2 in primitive VE and by inducing expression of the transcription factors forkhead box A2 (*Foxa2*) and LIM homeobox protein 1 (*Lhx1*). At E5.5, DVE, a subset of VE at the distal tip of the embryo, responds to Nodal-Smad2 signaling by expressing a subset of genes that includes *Hhex* (Brennan et al., 2001; Mesnard et al., 2006). Nodal up-regulates its own expression through a Smad-Foxh1 dependent mechanism. Smad-Foxh1 also activates a negative-feedback circuit by inducing the production of extracellular antagonists of Nodal signaling *Cer1*, *Lefty1* and 2, as well as Wnt antagonist *Dkk1* in the DVE. These antagonists effectively inhibit Nodal and Wnt signaling in the EPI and restrict Nodal target gene activation to the most proximal regions, while maintaining the anterior character of the distal epiblast cells. In contrast, signaling molecules *Fgf5* and *Fgf8*, T-box transcription factor *T* (*brachyury*) and *Tdgf1* are restricted to proximal epiblast. Restricted Nodal-Smad2 and Wnt- $\beta$ -catenin signaling thus activate or repress discrete target genes in the DVE and generate P-D polarity (see Figure 2).

Radial symmetry of the mouse embryo is disrupted with the formation of anterior visceral endoderm (AVE), the first clear mark of an A-P polarity in the embryo. Until recently, it was believed that migration of the DVE to the anterior side of the embryo established the A-P axis at E6.0 in response to Nodal signaling. Lineage tracing studies have recently indicated that AVE is not derived from DVE. Instead, it is newly formed after E5.5 from *Lefty1* deficient visceral endoderm cells that move to the distal tip of the embryo. It is however still believed that DVE helps guides the migration of AVE by initiating the global movement of visceral endoderm cells (Takaoka et al., 2011). The AVE expresses genes such as *Goosecoid* (*Gsc*)

(Filosa et al., 1997), orthodenticle homologue 2 (*Otx2*) (Ang et al., 1994), *Hhex* (Thomas et al., 1998), *Lhx1* (Perea-Gomez et al., 1999), *FoxA2* (Filosa et al., 1997), *Cer1* (Belo et al., 1997), *Dkk1* (Glinka et al., 1998), *Nodal*, and *Lefty1* (Mesnard et al., 2006). *Otx2*, which is a downstream target of Smad2-induced *Foxa2* complexes formed in the VE, is also essential for axis rotation. In *Otx2*<sup>-/-</sup> embryos, visceral endoderm proliferation is not affected, but the morphogenetic movement of the visceral endoderm and the expression of *Lefty1* and *Dkk1* are ablated (Perea-Gomez et al., 2001). AVE formation thus effectively repositions the source of Nodal and Wnt antagonists. In addition to signals from within the visceral endoderm, signals from the prospective posterior part of the ExE are responsible for restricting the size of the AVE. Removal of the ExE results in an expansion of *Cer1* gene expression, which can be counteracted by posterior extra-embryonic cells transplanted to the vicinity of the AVE precursors (Richardson et al., 2006).

Finally left-right asymmetry is established primarily through Nodal signaling. In the cylindrical-shaped mouse embryo, *Fgf8* induces the expression of Nodal on the left side (Dorey and Amaya, 2010), but during primitive streak induction, *Lefty* and other Nodal antagonists confine Nodal activity to the posterior end of the embryo. The result is a gradient of Nodal activity that is high on the ventral side of the embryo. The function of Nodal however declines as a gradient to the midline through antagonism. This asymmetry has been confirmed through genetic studies showing that mutations in *Acrv2b*, *Criptic* (*Cfc1*), and *FoxH1* in mouse all affect left-right axis formation. Mouse embryos mutant for the Notch ligand *Dll1* or doubly mutant for *Notch1* and *Notch2* also exhibit multiple defects in left-right asymmetry. *Dll1*<sup>-/-</sup> embryos do not express Nodal in the region around the node. *Dll1*-mediated Notch signaling is therefore essential for generation of left-right asymmetry, and peri-node expression of Nodal is an essential component of left-right asymmetry determination in mice (Krebs et al., 2003).

Despite known molecular asymmetries in the expression of several components of the Wnt, Fgf, and Nodal signaling cascades, the identity of the earliest determinants of axis polarity in the developing embryo are still only partially understood. Despite this, the combination of a proximally localized source of Nodal activating *Furin* and *Spc4* genes from the ExE (Beck et al., 2002) and *Lefty1*, *Cer1* and *Dkk1* genes at the distal/anterior end of the embryo (Perea-Gomez et al., 2002) result in a proximal-distal gradient of Nodal signaling with highest expression at the extra-embryonic/epiblast boundary (Lu and Robertson, 2004). This results in a mouse embryo that is pre-patterned by regional differences in gene expression. Morphological and radial symmetries are maintained until the onset of gastrulation, but by E6.5, the embryo is no longer symmetrical and it has both a short and a long axis.

## GASTRULATION

Gastrulation, the process during which time the three germ layers are specified, begins with the formation of the primitive streak on the posterior side of the embryo (diagonally to the AVE) at the junction of the ExE and epiblast. The primitive streak is the first overt morphologically distinct structure to break the bilateral symmetry of the embryo. It then elongates towards the distal tip of the embryo between the visceral endoderm and epiblast. Effectively it is an inductive tissue that produces signaling molecules like Wnt3, Bmp4 and Nodal, and it is the point at which most epiblast cells under an epithelial to mesenchymal transition (EMT) emerge as either mesoderm or endoderm.

### Initiation of Primitive Streak Formation

Prior to gastrulation, epiblast cells are not restricted in their lineage potency prior to recruitment and ingression. In chimeric embryos containing *Nodal*<sup>-/-</sup> mESCs, *Nodal*-deficient cells preferentially populate the anterior compartment of the epiblast, suggesting that cell mixing in the epiblast is not random and Nodal signaling mediates a cell-sorting process within the epiblast before gastrulation (Lu and Robertson, 2004). Clonal analysis also shows that epiblast cells are not restricted to their lineage potency prior to recruitment and ingression. At ~ E6.0, these cells begin to converge towards the posterior proximal pole of the embryo to form primitive streak, which is located on one side of the short axis. Only after ingressing through (mesoderm or endoderm) or by-passing (ectoderm) the primitive streak do cells of the EPI display highly restricted lineage potency.

Germ layer emergence from the primitive streak occurs through an orderly process. Nascent mesoderm is formed first when epiblast cells ingress at the primitive streak and undergo an EMT. These cells emerge from the primitive streak to form a new cell layer between epiblast and overlying the visceral endoderm that moves distally and laterally to eventually surround the entire epiblast. The earliest, most posterior mesoderm subpopulations give rise to the extra-embryonic tissues that include the mesodermal layer of the chorion and visceral yolk sac, and blood islands. Lateral plate, paraxial and cardiac mesoderm emerge from the intermediate and anterior portions of the primitive streak. At the anterior end of the primitive streak specialized organizer structures form progressively, known as the early gastrula organizer (EGO), mid gastrula organizer (MGO) and the node (Kinder et al., 2001). The EGO and the MGO generate the axial mesendoderm and definitive endoderm (DE) that initially forms the gut tube (foregut, then midgut, and finally hindgut precursors). DE also gives rise to lungs, liver, pancreas and thyroid. The node establishes the left-

right axis, and cells emanating from the node form the axial mesodermal structures, which consists of mesoderm that will populate the midline of the embryo (prechordal plate mesoderm, notochord) and signal patterning in the embryo.

The mesendoderm lineage remains a topic of debate, as its definitive characterization in the mouse embryo has not been completely resolved (Tada et al., 2005). The concept of a common mesendoderm progenitor has however gained momentum. In the mouse anterior primitive streak, presumptive mesodermal derivatives and the definitive endoderm progenitors are all located in close proximity as they ingress through the anterior streak. The close proximity of these lineages in mouse primitive streak is consistent with the concept established in the other model systems (i.e., zebrafish and *Xenopus*) where mesoderm and endoderm are generated from a bipotent mesendodermal population (Kimelman and Griffin, 2000). The presence of mESC-derived mesendoderm has been identified by the production of endodermal cell types from T-positive cell populations that display mesoderm potential (Kubo et al., 2004). The presence of mesendoderm has also been observed after the addition of activin, Fgf2 and Bmp4 to hESC cultures (Vallier et al., 2009). Fate mapping studies in the mouse embryo furthermore demonstrate that the order of and site of progenitor cell ingression through the primitive streak will determine the fate of the mesendodermal cell types (Kinder et al., 1999; Tam and Beddington, 1987), and although mesoderm and endoderm ingress through the primitive streak simultaneously, the two tissue layers migrate around the epiblast independently. It is noteworthy that not all mesoderm is derived from the mesendoderm. Non-mesendodermal mesoderm cells are predominantly fated to form somatic tissue/body muscle, while mesendodermal mesoderm is fated to become lineages such as blood and heart (Warga and Nusslein-Volhard, 1999). If in fact, precursors of mesendoderm, as opposed to only mesoderm and endoderm, are present in mouse, then the mechanisms that guide their segregation into distinct cell lineages are still poorly understood.

## **Signaling During Gastrulation and Cell Lineage Allocation**

Concomitant with these morphological changes and cell migrations, active Nodal and Wnt signaling in the posterior regions of the embryo is necessary for gastrulation. Loss of Nodal signaling, for example, leads to an absence of anterior primitive streak derivatives such as the axial and paraxial mesoderm and definitive endoderm (Beck et al., 2002; Hoodless et al., 2001; Yamamoto et al., 2004). In contrast, elevation of Nodal signaling (due to down-regulation of a co-repressor or up-regulation of a Nodal antagonist) results in the over-expression of mesodermal markers (Meno et

al., 1999; Perea-Gomez et al., 2002). Moreover, inhibition of Fgf signaling is thought to affect Nodal signaling. At least in chick, expression of a dominant negative receptor, a Fgf receptor inhibitor or depletion of Fgf ligands inhibits primitive streak formation and mesoderm formation.

The formation of different populations of mesoderm and endoderm ultimately depends on the levels of protein, duration, and locations of Nodal and Wnt signaling activity (Ben-Haim et al., 2006). At E7.5, expression of *Bmp4*, *Wnt3* and *Nodal* is restricted to the posterior side of the gastrulating embryo, while inhibitors of Nodal and Wnt signaling (*Cer1* and *Dkk1*) are selectively expressed on the anterior side. This expression pattern effectively establishes signal gradients that differentially activate transcriptional regulators that confer positional information and initiate cell fate commitments. Nodal signaling through Smad2/3 patterns the middle primitive streak derivatives (Dunn et al., 2004) as selective loss of *Smad2* in the epiblast results in a failure to specify the anterior mesoderm and endoderm (Vincent et al., 2003). *Foxa2*, *foxx1* and *Eomes* genes are also thought to be primary targets of Smad2 and Smad4 signaling. *Eomes* is restricted to the anterior streak, chorion and nascent mesoderm; however, it is not required for initial AP patterning, but is required for the expression of *T*, *Fgf8*, *Wnt3* and other proximal-posterior genes (Russ et al., 2000). *Foxa2*, a down-stream target of Smad-Foxh1, is restricted to derivatives of the anterior mesendoderm, the pre-chordal plate and the anterior midline endoderm.

Nodal also directly up-regulates some genes like *Mixl1*, and in a positive feedback loop with Wnt/ $\beta$ -catenin, it can up-regulate genes encoding *T* (*brachyury*), *Tdxf1*, and *Fgf8* (Morkel et al., 2003). *T* is expressed throughout the entire primitive streak, the node and the notochord, but in *Wnt3a*<sup>-/-</sup> embryos, *T* is completely absent from the anterior half of the primitive streak (Arnold et al., 2000; Yamaguchi et al., 1999). *Mix1* homeobox-like 1 (*Mixl1*) is restricted to the intermediate primitive streak.

## LINEAGE SPECIFICATION

### Ectoderm

In mouse, ectodermal precursors are localized primarily at the anterior and distal regions of the egg cylinder stage embryo, and fate-mapping studies reveal that the epiblast (embryonic ectoderm) of the 6.5-day embryos during early gastrulation contains ectodermal derivative precursor cells. Specific populations of embryonic ectodermal cells can be mapped to different segments of the neural tube. Quinlan et al. (Quinlan et al., 1995) showed that the most anterior cells of the distal tip colonize fore-, mid- and hindbrain and contribute to non-neural ectoderm cells of the amnion and



craniofacial surface ectoderm. Cells in the most distal region of the epiblast also contribute to the three brain compartments, as well as to the spinal cord. Cells located at the posterior site of the distal cap are mainly localized to the caudal parts of the neural tube. Epiblast cells located outside the distal tip make only a minor contribution to neuroectoderm. Instead these cells give rise to surface ectoderm and other non-ectodermal derivatives, with only a minor contribution to the neuroectoderm. At day E7.5 during the late-primitive-streak-stage at 7.5 days, over 80% of cells in the embryonic ectoderm are destined to form the neural tube.

The fact that epiblast cells fated to form ectoderm do not ingress through the primitive streak suggests that the default state of EPI is differentiation to ectoderm or neuroectoderm (NE). This assumption is supported by several lines of evidence. Camus et al. (2006) showed that neural specification and regional identities characteristic of forebrain occur prematurely in *Nodal*<sup>-/-</sup> mutants. These same Nodal-deficient epiblast cells when explanted and cultured readily differentiate into neurons. The initial steps of neural specification and forebrain development must therefore take place before gastrulation in the mouse, and Nodal must have a role in the inhibition of anterior and neural fate determination during pre-gastrula stages. Consistently, loss of either *Cer1* or *Lefty1* on the anterior side of the epiblast disrupts NE specification, while loss of both of these antagonists results in an expansion of mesoderm at the expense of NE. Sustained expression of *Cer1* and *Lefty1* in the anterior DE and the midline mesendoderm during gastrulation maintains the overlying NE. Loss of Bmp Receptor 1A (*Bmpr1A*) also causes early neuronal differentiation and premature loss of pluripotency within the epiblast (Arnold and Robertson, 2009).

Dorso-ventral patterning of the developing neural tube also depends on Wnt signals. This is achieved by the counteracting activities of morphogenetic signaling gradients set up by Sonic Hedgehog (Shh) in the ventral floor plate and notochord, and the canonical Wnt/ $\beta$ -catenin pathway acting in the roof plate, the dorsal most region of the neural tube. While evidence that Wnt and Sonic hedgehog are direct antagonists of one another remains to be determined, the role of Wnt in patterning the neural tube is thought to work in an indirectly inhibitory manner towards Sonic Hedgehog via the canonical Wnt pathway.

## **Mesoderm**

Depending on the time and site of ingression at the primitive streak, distinct mesodermal cell lineages form, including lateral plate, paraxial and cardiac mesoderm. The lateral plate mesoderm forms the circulatory system and the gut wall while paraxial mesoderm forms the somites, the vertebral column and the skeletal muscle (Beddington and Robertson,

1999; Kinder et al., 1999; Tam et al., 2007). Developmentally, EMT allows nascent mesoderm to delaminate and migrate from the primitive streak. This process is associated with a loss of cell adhesion with basement membrane and changes in the cells's cytoskeletal structure. As up-regulation of *Cdh1* is associated with morula compaction, down-regulation of this protein is required for disruption of the epithelial adherens junctions. Through the use of loss-of-function mutants, the signaling cascade responsible for *Cdh1* gene repression during nascent mesoderm has been determined. Down-regulation occurs in response to Fgf signals through the Fgfr1 and induction of the zinc-finger transcriptional repressor Snail. More specifically, loss of Fgfr1 disrupts gastrulation, while Fgf8-deficient epiblast cells ingress at the primitive streak but fail to migrate away from the primitive streak. This effectively blocks both mesoderm and definitive endoderm formation. Snail mutants, however, develop nascent mesoderm that retains an epithelial-like morphology, but fail to down-regulate *cdh1* expression, which interferes with EMT. In mutants lacking *Eomes*, the induction of mesoderm markers is not compromised; however, nascent mesoderm fails to delaminate and migrate away from the primitive streak. This defect is also due to an inability of the cells to down-regulate *Cdh1* gene expression and undergo an EMT transition. Surprisingly, a lack of *Eomes* does not affect *Fgf8* or *Snail* gene expression, suggesting that *Eomes* acts independently of Snail. Thus, *Eomes* is also critical to EMT and mesodermal cell migration (Arnold and Robertson, 2009).

Fgfs control the specification of and maintenance of mesoderm rather than as an inductive factor of primary mesoderm (Burdal et al., 1998; Ciruna and Rossant, 2001). Mutational analysis of the known *Fgf* genes have demonstrated that only *Fgf4* and *Fgf8* are required for early embryonic development (Ciruna and Rossant, 2001; Crossley and Martin, 1995; Niswander and Martin, 1992). *Fgf8*<sup>-/-</sup> embryos fail to express *Fgf4* in the primitive streak and in the absence of both Fgf8 and Fgf4 epiblast cells move into the primitive streak and undergo an EMT but then fail to move away from the primitive streak (Sun et al., 1999). Fgfr1 is expressed throughout the epiblast prior to gastrulation and becomes restricted to the posterior/primitive streak as gastrulation proceeds (Yamaguchi et al., 1992). Fgfr1 has a role in mesoderm cell fate and is required for *T* and *Tbx6*, a marker of paraxial mesoderm (Chapman et al., 2003), expression in the primitive streak. Fgfr1 orchestrates the epithelial to mesenchymal transition at the primitive streak by up-regulating *Snai1* which functions to repress *Cdh1*. Fgfr1 also functions indirectly in mesoderm/endoderm cell fate specification, since down-regulation of *Cdh1* (which has a potent ability to sequester  $\beta$ -catenin) allows the Wnt/ $\beta$ -catenin pathway to positively regulate *T* (*brachyury*) and *Tbx6* expression (Ciruna and Rossant, 2001). *Tbx6* has also recently been shown to determine neural or mesodermal fate

in axial stem cells present in early gastrulation. It functions by repressing *Sox2* activity to inhibit neural development, thus specifying paraxial mesoderm (Takemoto et al., 2011). *Pax3* has also been implicated in myoblast determination (Buckingham, 2007). Finally, mesoderm posterior 1 (*Mesp1*) is expressed in a subset of mesodermal cells and is thought to be a marker of cardiac progenitor cells. Although expression of *MESP1* depends on Notch signaling and disappears before E8.5, it is also regulated by *Eomes*. *Eomes* is an upstream regulator of *Mesp1*, but in contrast to *Eomes*/*Nodal* signaling interactions that cooperatively regulate anterior–posterior axis patterning and allocation of the definitive endoderm cell lineage, formation of cardiac progenitors requires low levels of *Nodal* activity (Costello et al., 2011).

## Definitive Endoderm

The endoderm is the innermost germ layer that makes up the embryonic body plan; and consequently, endoderm morphogenesis poses a unique hurdle to the embryo. Endodermal precursor cells from epiblast have a reversed polarity to the one that they will have in the definitive endoderm. Prospective DE cells therefore must insert into the surface epithelial layer of the embryo and undergo an apical-basal polarization soon after EMT at the streak. It is generally believed that DE cells may already be specified within the streak and that they polarize as they insert into the visceral endoderm epithelium at the surface (Zorn and Wells, 2007).

Once endodermal cells emerge from the anterior end of the primitive streak, the cells migrate into the outer layer of cells on the embryo. Two models have been proposed to explain how endoderm emerges (Tremblay, 2010). The first is a displacement model, whereby DE emerges and displaces VE towards the proximal extraembryonic region. The second model suggests that the DE layer is produced by the intercalation of cells from the epiblast into existing VE. This would cause a transient salt and pepper distribution of the VE, but thus far neither transcript or protein expression data have demonstrated intermingling of visceral and definitive endoderm. Regardless of the model, DE and VE are in contact with each other from the onset of embryonic endoderm emergence.

One recurring principle that has emerged is that *Nodal* signaling is critical for promoting endoderm. More specifically, high versus low levels of *Nodal* are required for endoderm versus mesoderm formation, respectively. In mouse, *Nodal*-*Smad2* activates and/or maintains the expression of critical transcription factors and *Nodal* ligands necessary for endodermal fate decisions. This complex activates *Eomes* as well as *SRY* box (*Sox*), *Gata* and *Mix*/*Bix*-type homeobox genes families to regulate definitive endoderm formation. Mouse knockout data indicate that *Sox17*, *Mixl1*, and *Eomes* also regulate distinct and overlapping aspects of endoderm formation. In

mouse Sox17 is not necessary for definitive endoderm specification, but it is required to promote endoderm survival. In human ESCs, however, Sox17 drives differentiation to predominantly definitive endoderm. Mix-like proteins appear to promote endoderm fate decisions while suppressing mesoderm gene expression, while an absence of Eomes expression in epiblast causes the formation of an embryo that entirely lacks definitive endoderm. Hepatocyte nuclear factors (Hnfs), which may be regulated by Nodal signaling, are also involved in the initiation and maintenance of the endodermal lineage. In mouse, Foza2 (previously known as Hnf3 $\beta$ ), a factor expressed in the anterior region of the primitive streak, in endoderm and the early liver, is essential for the development of prospective foregut and midgut endoderm. Several other transcription factors are also required for endoderm patterning and organ development. Nkx2-1 is required for thyroid development and lung morphogenesis. Hhex is essential for liver and thyroid development, and Pdx1 is required for the formation of the ventral and dorsal pancreas (for references, see (Kubo et al., 2004)).

## **Summary of Early Developmental Processes**

Signaling pathways, cell adhesion properties and transcription factor regulation function together to specify cell types of all lineages both during pre- and post-implantation stages of embryo development. Nodal in conjunction with Wnt, Notch, and Fgf signaling establish gradient effects that modulate the external environment. In response to these gradients, cells differentially regulate their own gene expression to induce or repress transcription factors that in conjunction with the signaling molecules control early fate decisions like TE versus ICM. ICM subsequently forms two other lineages with more restricted differentiation potential. In cells destined to become PE, Gata6 promotes endoderm formation; while Oct4, Nanog and Sox2 drive EPI specification. The EPI, in particular, maintains a high degree of plasticity and can generate all somatic and germ cells of the embryo proper. As EPI cells either ingress or by-passes the primitive streak their fate choices become limited, and ultimately new signaling events, changes in cell adhesion, and the activation of defined transcription factors lead to determination events that allow formation of a viable fetus and adult with functioning tissues and organs.

## **ESC DIFFERENTIATION**

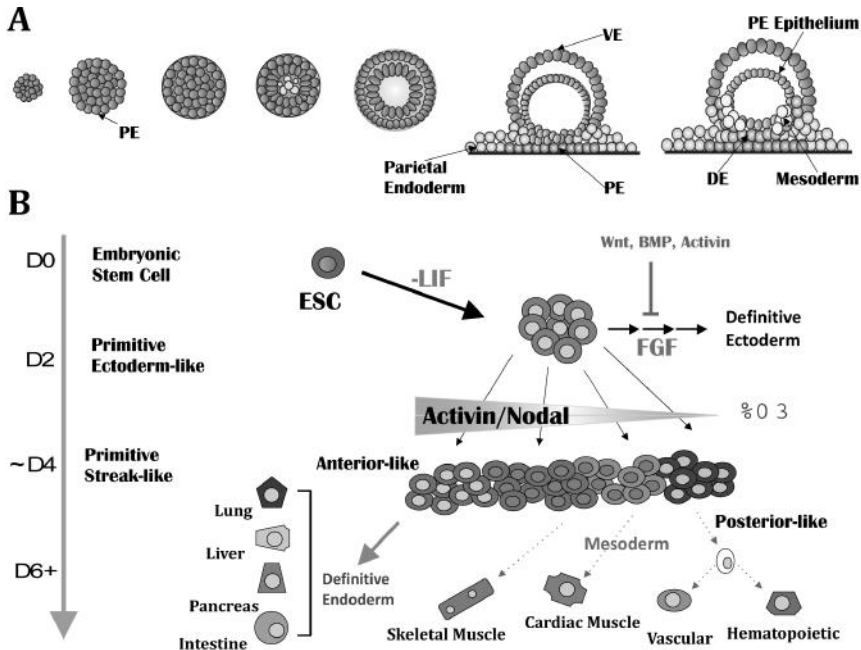
ESC differentiation is quite different from embryo development. As just described, correct cell specification requires signal gradients with appropriate spatial and temporal regulation of transcription factors that

ultimately permit cell determination. Although ESCs have a high degree of plasticity similar to that seen with EPI and similar events can happen *in vitro*, our inability to fully model *in vivo* development often prevents the generation of fully functional and mature stem cell progeny. This limitation is a major hurdle to regenerative medicine but, by understanding some of the limitations associated with *in vitro* differentiation, it is likely that we will be able to improve this system to make therapeutic application of pluripotent stem cells and their progeny a reality.

## Mouse

ESCs are isolated from the ICM or EPI of pre-implantation embryos, and when differentiated *in vitro* can also form cells of all three primary germ layers. A commonly used strategy for directed *in vitro* differentiation of mouse (m) and human (h) ESCs involves an aggregation step in suspension that results in the formation of embryoid bodies (EBs). This system, as opposed to cells differentiated as monolayers, more closely resembles pre-implantation development in a fluid-like environment, and it recapitulates many aspects of early post-implantation embryonic development. The process also mimics the ontogeny of gene expression patterns that appear during embryogenesis. Structurally, mEBs are 3-dimensional, spherical multi-cellular aggregates that differentiate in a spontaneous, predictable manner (Weitzer, 2006). The early stages of mEB development can be separated into seven distinct stages (described below): (1) aggregation, (2) primitive endoderm formation, (3) basement membrane assembly, (4) primitive ectoderm epithelium formation, (5) cavitation, (6) visceral and parietal endoderm formation and (7) germ layer induction (Figure 3). While gastrulation-like processes and germ layer induction take place in mEBs (Weitzer, 2006), the process has intangible variation (i.e., developmental noise) that is highly dependent upon cultivation techniques, including appropriate starting numbers of cells, and stochastic events.

When placed in suspension, individual mESCs aggregate to form an irregularly shaped cluster of cells that are morphologically indistinguishable and mimic an early morula-like structure (Murray and Edgar, 2004; Wobus and Boheler, 2005). By day 2 in suspension, the ESCs contained in the EB proliferate, and the mEBs have “bubbles of cells” on the surface, which subsequently form a smooth cell monolayer over the entire surface of the mEB. This resembles the process of morula compaction that occurs during development. An essential factor in the aggregation process of mEB formation is Cdh1 (E-cadherin), a calcium-dependent cell-cell adhesion glycoprotein. Developmentally, E-cadherin is first expressed in the 2-cell staged mouse embryo. It is phosphorylated by the 8-cell stage, and it has been implicated in morula compaction. Mouse ESCs lacking *Cdh1* (*Cdh1*<sup>-/-</sup>)



**Figure 3.** (A) Seven stages of EB formation and development. Day 1, Cdh1 mediated mESC compaction (blue cells). Days 2–4, primitive endoderm formation on the exterior of the mEB (red cells). Days 3–5, basement membrane assembly (grey). Days 4–5, primitive ectoderm epithelium formation and programmed cell death of epiblast-like cells not in contact with basement membrane. Days 5–6, continued development of primitive ectoderm epithelium, visceral ectoderm development and of cavitation completion. Day 7, mEB attachment to a plate or collagen matrix (black line) via primitive endoderm (red cells underneath the centre of the mEB) which spreads away from the mEB to form parietal endoderm (green cells). Visceral endoderm forms epithelial cysts on top of the central cell mass of the mEBs (red cells above centre of mEB). Days 8+, Primitive ectoderm epithelium (blue cells) differentiate to mesodermal cells (yellow) and definitive endoderm (purple). The culture medium substitutes for the blastocoel, and the central cyst (inside primitive ectoderm epithelium) is analogous to the pro-amniotic cavity in the embryo (diagram adapted from Li et al., 2003; Rodda et al., 2002; Weitzer, 2006). (B) Probable scheme for ESC differentiation and the role of specific factors in the formation of primitive ectoderm and primary germ layers through the generation of a hypothetical primitive streak-like structure in EBs. The formation of this hypothetical primitive streak-like structure should permit posteriorization and anteriorization of cell populations. Consistent with *in vivo* development, Fgf induces neural development independent of primitive streak formation, while Wnt, Bmp and activin are antagonistic. Bmp4 also functions to induce posterior mesoderm, and as in the primitive streak, Nodal/Activin signalling is concentration dependent. This figure has been adapted from (Perino et al., 2008).

Color image of this figure appears in the color plate section at the end of the book.

do not form EB aggregates and fail to undergo differentiation (Larue et al., 1994). Moreover, *Cdh1*-dependent aggregation may be a prerequisite for the restriction of Fgf signaling to the outer cells of the developing mEB and primitive endoderm formation.

By day 4–5, cells continue to emerge and elongate until the entire surface of the mEB is covered by extra-embryonic-like PE (Smyth et al., 1999). These cells are characterized by the expression of *Afp* ( $\alpha$ -fetoprotein), *Nrtf1* (Coup-Tf1) and *Krt8* (cytokeratin 8), and an absence of *Nanog*. The outer layer of PE express basement membrane components, consisting largely of laminins and collagen Type IV (reviewed by Li et al., 2003). Concomitantly, undifferentiated mESCs in the center of the mEB form primitive ectoderm and EPI-like cells. This is associated with up-regulation of *Fgf5* and down-regulation of *Bmp4*. EPI-like cells in contact with the basement membrane become polarized and undergo primitive ectoderm epithelialization, perhaps in response to Bmp and Indian hedgehog (*Ihh*) signaling from the PE.

Analogous to the earliest wave of programmed cell death that occurs during embryogenesis, cavitation begins when cells located in the center of the mEB die via apoptosis and autophagy, which is mediated in part by apoptosis inducing factor (*Aif*) and oxidative phosphorylation. Concomitantly, PE on the outer surface of the EB differentiates to visceral endoderm, which is characterized by columnar epithelial morphology and expression of markers like HNF4A (Hepatic nuclear factor 4,  $\alpha$ ), *Foxa2*, *Sox17*, *Afp*, *Evx1*, *Ttr* (transthyretin) and *Krt8*. By day 4–5 post-aggregation, the mEB is composed of an inner epiblast and an outer endoblast, with the medium functioning as a substitute for the blastocoel and the inner cavity as a surrogate for the pro-amniotic cavity. Mouse EB differentiation in suspension thus recapitulates hypoblast development to produce PE that gives rise to visceral and parietal endoderm. The latter subsequently undergoes an epithelial to mesenchymal transition to form mature mesenchymal parietal endoderm, which provides the foundation for gastrulation-like development. Mechanistically, *Bmp4* has been implicated both in cavitation and visceral endoderm formation as inhibition of Bmp signaling prevents *Hnf4a* expression, cavitation and visceral endoderm formation. Indian hedgehog (*Ihh*) signaling also has a role in visceral endoderm formation, since downstream inhibition of *Ihh* signaling promotes the outer PE to form parietal endoderm instead of visceral endoderm (Maye et al., 2000).

When transferred onto tissue culture plates, PE-derived basement membrane allows the mEB to attach. The remaining PE in contact with the dish forms a sheet of parietal endoderm (Grabel and Casanova, 1986) that migrates away from the central portion of the mEB that subsequently undergoes an epithelial to mesenchymal transition (Bader et al., 2001). This

transition is characterized by up-regulation of *Plat* (tissue plasminogen activator) and *Krt19* (cytokeratin 19), and down-regulation of *Afp* (Stary et al., 2005). After attachment, visceral endoderm is maintained as an epithelial bubble on top of the primary cell mass of the mEB (Bader et al., 2001; Murray and Edgar, 2001). Within 2 days of attachment, primitive ectoderm epithelium inside the ring of visceral and parietal endoderm begins to differentiate towards the three definitive germ layers: endoderm, mesoderm and ectoderm.

Gastrulation-like processes are initiated by up-regulation of markers for mesoderm, definitive endoderm and definitive ectoderm; however, morphological cell movements that occur during developmental gastrulation do not occur in a highly reproducible manner. Under some conditions, mEBs have been described that show rudimentary morphogenetic development, where an epithelial sheet of cells is positioned exclusively on one side of the mEB, thus establishing a degree of axis formation. The development of mesodermal cells in a restricted area of the mEB, between the primitive endodermal and ectodermal layers, also suggests that the axis formation may mimic aspects of the primitive streak stage of mouse development. These symmetries and axes are not highly reproducible and are not observed with mEBs plated at high densities. Mouse EB development is therefore thought to be primarily stochastic in nature.

Despite the chaotic nature of differentiation, mEB primary germ layer formation recapitulates some of the signaling pathways experienced *in vivo*. In fact, EBs are thought to express most if not all of the growth factors, signal mediators and transcription factors necessary for the regulation of embryogenesis *in vivo* (Irion et al., 2008; Weitzer, 2006). The parietal endoderm has been shown to influence the primitive ectoderm epithelium to differentiate towards mesodermal lineages, as demonstrated by the detection of a proliferating horseshoe shaped area of mesoderm, that expresses T (Brachyury), inside the ring of parietal endoderm which goes onto form erythrocytes and cardiomyocytes (Bader et al., 2001). Importantly, the chaotic nature of germ layer differentiation in mEB is likely due to the lack of polarity and the lack of trophoblast and subsequent extra-embryonic ectoderm (Weitzer, 2006). The stochastic patterning and developmental noise that occurs may also be caused by (dys)regulated gene expression, mosaic patterns of gene expression that occur in mEBs, and probable epigenetic modifications due to culture and clonal line variations.

A possible scheme for ESC differentiation and the role of specific factors in the formation of primitive ectoderm and primary germ layers is shown in Figure 3. Here a hypothetical primitive streak consisting of both posterior and anterior cell populations is thought to develop *in vitro* consistent with what occurs in mouse. Neuroectoderm induction bypasses the primitive streak, and relies on FGF signaling. As with development,



other signaling pathways, including those for Wnt, BMP, and activin are all implicated as regulators of ectoderm induction. In ESCs, BMP4 functions to induce posterior mesoderm, while a gradient of activin/nodal signaling controls fate decisions associated with definitive endoderm and mesoderm induction. Based on *in vitro* studies, low concentrations of activin generate, more posterior-like populations; whereas high concentrations of activin induce endoderm, consistent with an anterior primitive streak-derived population described in mouse. Taking advantage of this knowledge, it is possible to preferentially differentiate ESCs into one of the three primary germ lineages, merely by altering the concentrations of inductive or repressive factors (Wnts, Nodal, etc.). While useful for cell commitment, the resulting progeny are not however always as mature or functionally viable as mature cells generated *in vivo* (see Figure 3).

## Human

Differentiation processes similar to those that occur in mouse EBs, including radial differentiation, apoptosis, central cavitation and expression of primitive endoderm associated markers, have been reported in hEBs. Ikskovitz-Eldor et al. (2000) described human H9-derived EBs as being composed of densely packed cells at day 3 before becoming cavitated at day 4, accumulating fluid and becoming cystic at day 14 (with between 20–90% of hEBs cystic at day 20). Cystic hEBs are hEBs that have developed a fluid filled cavity analogous to that present in a developing blastocyst. Conley et al. (2007) described HES-2 hEBs as containing fluid-filled cystic EBs from day 7 onwards and observed the presence of apoptotic cells at days 3, 5 and 7. Since apoptosis in hEBs also occurs before the formation of a visceral endoderm layer, this cell lineage may not be required for the initiation of cavitation in the hEB system (Conley et al., 2007). The presence of primitive endoderm has been described in HES-2 hEBs by the detection of *Afp*, *Krt8*, and *Gata6* expression from days 3–5 (Conley et al., 2007). At day 7 expression of these markers was detected in a thin layer of cells at the hEB periphery, suggesting differentiation of primitive endoderm to visceral endoderm (Conley et al., 2007). Primitive endoderm marker expression in an outer epithelial layer has also been described in day 8 H1 hEBs by the detection of *Krt8* (Gerami-Naini et al., 2004) and in day 13 HSF6 hEBs by the detection of *Afp* (Poon et al., 2006). Finally, hEBs formed from H9 or Hsf6 hESCs differentiated in conditioned media grow as homogenous spheres with no apparent tissue organization at Day 10 and neuroectoderm-like differentiation at days 11–14 (Vallier et al., 2004). In contrast, transgenic over-expression of *nodal* induced 75–95% of hEBs to form three distinct cell layers

at day 10. AFP was expressed in the outside layer and OCT4 was expressed in the inner layers, although no definitive germ layer differentiation was detected (Vallier et al., 2004).

## Mouse vs. Human

Undifferentiated mouse and human ESCs share several fundamental properties. ESCs from these two species have an absolute requirement for *Oct4*, *Sox2* and *Nanog* gene expression. In fact, these transcription factors in conjunction with a process known as reprogramming can reprogram otherwise determined somatic cells into induced pluripotent stem cells (iPSCs) that have most if not all of the characteristics of ESCs (Yamanaka, 2008). Moreover, both species of ESCs can differentiate into any type of somatic cell found in the body, but with some limitations. Neither human nor mouse ESCs generate definitive hematopoietic stem cells in culture. Instead only embryonic forms of HSCs have been generated. In contrast, neuronal cells, which represent the default state of EPI and by extension ESCs, can be readily generated, with properties similar to that seen in the adult. The ability to generate high quality, adult-like cells therefore seems to be dependent on the developmental stage of specification and determination for specific cell types.

Despite these similarities, there are significant differences between mouse and human development that may be reflected in EB differentiation. In humans, the first 10 days of embryonic differentiation are almost exclusively devoted to the formation of the extra-embryonic membranes whereas the mouse has already reached midgestation by this point (Dvash and Benvenisty, 2004). This suggests that *in vitro* hEB differentiation should be substantially slower than mEB differentiation. Also of particular relevance to hEB differentiation are the differences in the arrangement of the germ layers. Human embryos form a planar bilaminar disc, whereas mouse embryos form a more compact egg cylinder that undergoes germ layer-inversion (Eakin and Behringer, 2004). It has been suggested that mouse gastrulation, which is substantially more three-dimensional than human gastrulation may also have unique features compared to in other mammals going through gastrulation (Beddington and Robertson, 1998; Tam and Behringer, 1997). Also in the human embryo, extra-embryonic mesoderm is thought to be derived from the extra-embryonic endoderm before the appearance of the primitive streak (Bianchi et al., 1993).

It should be realized that hESCs and mESCs display several fundamental differences. First, the cell surface proteins used to characterize these cells differ. SSEA-1 is a marker of undifferentiated mouse ESCs, while SSEA-3 and SSEA-4 are found on hESCs. Mouse ESCs can be maintained in a undifferentiated state by cultivation with leukemia inhibitor factor (LIF);

however, the addition of LIF does not sustain the culture of hESCs in the absence of feeder layers (Thomson et al., 1998) despite the expression of both LIF and LIFR in hESCs. Activation of this signaling pathway (LIF receptor/GP130 complex) is therefore not fundamental to hESC pluripotency. Instead Tgf- $\beta$ /activin A/Nodal pathway activation and Bmp pathway repression by NOG (noggin) are required to maintain hESC pluripotency, while Fgf2 signaling through PI3K/Akt is required to maintain cellular proliferation. Recently however, co-expression of Rarg (Rar- $\gamma$ ) and Lrh-1 (liver receptor homologue 1; Nr5a2) with the four reprogramming factors (Oct4, Sox2, Klf4, Myc) successfully directed reprogramming of somatic cells towards ground-state pluripotency (i.e., epiblast-like) in both mouse and human (Wang et al., 2011). Importantly, these reprogrammed iPSCs, from both species, are LIF dependent. Thus, the derivation of ground-state EPI-like cells from mouse and human, which are functionally very similar, should offer novel insights into differentiation that will ultimately foster comparisons that will permit a greater understanding of human developmental processes.

Finally, conventional mESC are derived from the ICM, but it has recently been demonstrated that pluripotent cells can be derived from the post-implantation stage EPI, termed epiblast stem cells (EpiSCs). EpiSCs can self-renew and are pluripotent according to standard assays but are not able of incorporating into the ICM and contributing to the formation of a chimeric mouse, confirming that EpiSCs are from and represent a later developmental stage of pluripotency compared with ICM-derived mESCs (Brons et al., 2007). EpiSCs are cultured in hESC-type conditions (relying on TGF-B/activin/Nodal and FGF signaling) and have a hESC morphology, gene expression and signaling response (Tesar et al., 2007). Intriguingly, it has been demonstrated that EpiSCs can be converted to mESC by using a cocktail of small molecules (Zhou et al., 2010).

## CONCLUSIONS AND FUTURE PERSPECTIVES

Stem cells from various tissues have common attributes that enable them to self-renew, survive and produce progeny. As we have seen, pluripotent stem cells can generate derivatives of all three germ layers *in vitro*, but the differentiation process is highly chaotic. This is due to the lack of highly controlled and structured specifying signals *in vitro* that are present in the developing embryo. In the absence of these constraints and gradients, ESCs will differentiate preferentially to the cell type induced by the culture conditions. The types of cells generated are, however, often immature, and the degree of maturation appears to be correlated with the normal developmental profile that occurs *in vivo*.

The generation of mouse and human ‘ground-state’ iPSCs demonstrates that many hESC lines may not have been derived from the same developmental stage as their mouse counterparts. Other differences also suggest that hESCs are more similar to EPI cells of the early post-implantation embryo than mESC derived from pre-implantation embryos. Despite these differences, the use of hESCs and ‘ground state’ hiPSCs, are invaluable for the study of human development—as no other system is truly available that is readily manipulated with regards to gene expression and disease modeling. The analysis of human development based on *in vitro* differentiation models must, however, always take into account the spatial constraints associated with development. Moreover, if some human diseases are ever to be treated effectively with ESC/iPSC-based therapies, then it is likely that the use of *in vivo* developmental mimicry that results in a greater degree of maturation may be required. With that said, there is still a great deal that is unknown with regard to mouse and particularly human embryonic development, but as this field continues to move forward, it is also likely that other developmentally relevant stem or progenitor cells will be identified from these models that have greater value therapeutically. This is because these ‘less pluripotent’ cells may have already been specified or determined to form viable progeny that will improve the prospects of transplantation therapy.

## ABBREVIATIONS

|     |                                      |
|-----|--------------------------------------|
| TE  | trophectoderm                        |
| ICM | inner cell mass                      |
| EPI | epiblast                             |
| PE  | primitive endoderm                   |
| P-D | proximal-distal                      |
| A-P | anterior-posterior                   |
| ExE | extra-embryonic ectoderm             |
| VE  | visceral endoderm                    |
| DVE | distal visceral endoderm             |
| AVE | anterior visceral endoderm           |
| EMT | epithelial to mesenchymal transition |
| EGO | early gastrula organizer             |
| MGO | mid gastrula organizer               |
| DE  | definitive endoderm                  |
| NE  | neural ectoderm                      |

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# Hematopoiesis During Embryonic Development

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## SUMMARY

Blood cells are constantly produced from a pool of progenitors that ultimately derive from hematopoietic stem cells (HSC). In vertebrates, the hematopoietic system develops from two distinct waves or generation of precursors. The first occurs in the yolk sac, or equivalent embryonic structure, and produces nucleated primitive erythrocytes, that provide the embryo with the first transporter of oxygen and are therefore essential for the viability of the early embryo. The yolk sac also produces myeloid cells that migrate to the central nervous system and to the skin, to form the microglia and skin specific macrophages, the Langerhans cells. After a first period of expansion, these populations become resting and are found throughout adult life. The second generation occurs in the dorsal aorta and produces HSC. These are generated once in the lifetime, from mesoderm

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*List of abbreviations after the text.*

derivatives closely related to endothelial cells, during a short period of embryonic development. They develop in close contact with the endothelial lining of the aorta-gonad-mesonephros region. Before reaching the adult hematopoietic organ, bone marrow in most mammals, HSCs are transiently detected in an embryonic organ with equivalent function, the fetal liver. It is in the fetal liver that differentiation of HSCs first occurs giving rise to mature blood cells. HSCs also expand in the fetal liver and in a short time window (four days in the mouse embryo) they increase over 40-fold. HSCs and progenitor cells exit the fetal liver and colonize the spleen, where differentiation to the myeloid lineage and particular lymphoid subsets is favored. After reaching a maximum number in fetal liver, stem cells colonize the bone marrow, so that few are found in the liver after birth. In the bone marrow stem cells rapidly lose their proliferative capacity and the pool of HSCs remains stable throughout life, in steady state conditions. HSCs generate mature hematopoietic progeny while keeping constant the HSC pool due to their unique self-renewal capacity that is likely the product of asymmetric cell division.

## INTRODUCTION

In mammals, blood cells are constantly produced in the bone marrow through the expansion and differentiation of progenitors that originate from a rare cell population, the hematopoietic stem cell (HSC). HSCs are generated once in the lifetime during embryonic development and production of mature hematopoietic cells starts in the fetal liver (FL) and continues in the bone marrow (BM). We discuss here, the recent advances in understanding the mechanisms underlying the establishment of the hematopoietic system during embryonic development.

Bone marrow transplantation has been used for more than 80 years to treat hematopoietic disorders. It was not, however, until the early 60s that the hallmarks of hematopoietic stem cells, multipotency and self-renewability, were uncovered. The seminal work of Till and McCulloch set the basis for the *in vivo* assays that are still used today to identify stem cell activity (Till and McCulloch, 1961; Till et al., 1964). Their experiments were based on the capacity of injected bone marrow (BM) cells to undergo clonal expansion in the spleen of irradiated recipient mice. The founding colony progenitor was called colony-forming-unit-spleen (CFU-S). The results established that adult BM contained a population of cells that could give rise to several different blood lineages at the single cell level and therefore was a multipotent progenitor. The concept of self-renewability derived from the observation that single cells could give rise to colonies that contained both differentiated blood cells and new CFU-S progenitors functionally identical to the colony founder (Worton et al., 1969; Wu et al., 1968). These

studies also introduced the *in vitro* clonal assays as the “golden standard” to analyze differentiation potential. *In vitro* clonal cultures demonstrated that a subset of BM cells have multiple differentiation potentials (Metcalf, 1970; Moore and Metcalf, 1970). Such assays were also useful to identify and isolate specific soluble factors (hematopoietic cytokines) with essential roles in the development of individual blood lineages. In the 1980s, Keller and Snodgrass used retroviral vector integration as clonal markers and introduced the concept of long-term reconstitution (LTR) (Keller and Snodgrass, 1990; Snodgrass and Keller, 1987). They showed that the progenitor compartment in the BM was composed of cells that could either reconstitute the hematopoietic system for short periods of time (up to four months) or sustain the production of blood cells for the life of the recipient. Moreover, this long term reconstitution capacity could be serially transplanted, for up to four passages, in successive recipients. They thus demonstrated the *in vivo* self-renewing capacity of HSCs. Although these experiments provide compelling evidence for self-renewal of HSCs, they suffer from the criticism that the retroviral integration might have led to abnormal gene expression that could cause clonal dominance in the progeny of some cells (Kustikova et al., 2005). In summary, testing the functional properties of a heterogeneous population of BM cells led to the paradigm of multipotent and self-renewing adult stem cells.

Later, major efforts were concentrated in defining surface markers allowing prospective isolation of cells capable of long-term reconstitution of the hematopoietic lineage, in other words, the hematopoietic stem cells (HSCs). The Weissman laboratory contributed most to our current knowledge of progenitor phenotypes. HSCs were initially identified in the lineage (a cocktail of antibodies recognizing erythrocytes, myeloid cells including dendritic cells (DC), T, B and NK cells) negative (Lin<sup>-</sup>) BM compartment, expressing high levels of Kit (CD117) and Sca1 (Spangrude et al., 1988). Negative selection for CD34 and Flk2 (CD135) expression, allowed their further enrichment to efficiencies of 1:3–1:5 *in vivo* reconstitution assays (Osawa et al., 1996). More recently, HSCs were identified as CD244<sup>-</sup>CD48<sup>-</sup>CD150<sup>+</sup> cells (Kiel et al., 2005; Kim et al., 2006), a phenotype that is easier to define by flow cytometry and microscopy, and that allows a better definition of HSCs in histology. HSC isolated as Lin<sup>-</sup>Sca1<sup>+</sup>Kit<sup>+</sup>CD34<sup>-</sup>Flk2<sup>-</sup> or as CD244<sup>-</sup>CD48<sup>-</sup>CD150<sup>+</sup> are largely overlapping. The identification of surface markers that subdivide the progenitor compartment and defines HSCs is also important to allow the characterization of discrete, subsequent stages of differentiation, accompanying loss of self-renewing capacity and partial engagement in particular hematopoietic lineages.

In the last decade, regenerative progenitors have been identified and isolated in many tissues. When cultured in differentiating conditions or when injected *in vivo*, they give rise to cells from the tissue of origin, and

are therefore tissue specific. In adults, tissue regeneration is the property of tissue specific progenitors, and pluripotent cells with broader differentiation potential have not been identified. It appears that most, if not all, adult stem cell compartments are established during a defined period of time of embryonic development, during organogenesis. Tissue renewal is obtained throughout life by the expansion and differentiation of such progenitors, whereas the maintenance of the compartment is ensured by asymmetric cell division. We argue that understanding adult stem cell physiology requires the understanding of how the tissue was specified in fetal life.

## **EMBRYONIC HEMATOPOIETIC SITES**

### **The Yolk Sac**

The first hematopoietic cells of embryonic origin are present in the yolk sac (YS), starting at mouse embryonic day (E) 7.5. The YS blood islands, generated from condensed cellular aggregates of morphologically identical cells, harbor the first endothelial and hematopoietic progenitors. Blood islands fuse to form the vascular network of the YS. This sequence of events suggests that both endothelial and hematopoietic cells share a common progenitor, designated as the hemangioblast. Attempts to confirm this hypothesis and isolate this cell type have not been conclusive. Recent sophisticated genetic approaches (Ueno and Weissman, 2006) lead to the conclusion that each YS blood island is formed by more than one progenitor and contribution from single progenitors to both lineages was not consistently observed, suggesting that blood islands are formed by aggregation of distinct progenitors committed to the endothelial or hematopoietic lineages. The YS origin of HSCs was initially proposed, based on the unique early hematopoietic activity in this site. Following injection of YS cells in the embryonic circulation, T cells of donor origin were detected in the thymus of the recipient, bringing experimental evidence to the hypothesis of the YS origin of HSCs (Weissman et al., 1977). Pioneer experiments carried out in the avian model, however, indicated otherwise. Quail-chicken and congenic chicken chimeras showed that YS hematopoietic cells were only capable of transient contribution to the hematopoietic compartment (Dieterlen-Lievre, 1975; Dieterlen-Lievre et al., 1976; Lassila et al., 1978). In these chimeras, definitive hematopoiesis had an intra-embryonic origin. Additional evidence came from experiments done in *Xenopus* where YS (ventral blood islands) and intra-embryonic blood compartments (dorsal lateral plate) were derived from different blastomeres of 32-cell embryos and therefore independently generated (Ciau-Uitz et al., 2000). Although the experiments in chicken, *Xenopus* and later in the mouse demonstrate that HSCs have an intra-embryonic

origin, recent lineage-tracing experiments argued for a contribution of YS to definitive hematopoiesis. The experiment relies on permanently labeling cells irrespective of their location, based on the expression of a common endothelial and hematopoietic transcription factor—Runx1 (Samokhvalov et al., 2007). However, these experiments do not conclusively rule out early intra-embryonic generation of HSCs being detected.

Initially, YS blood islands contain erythrocytes from the primitive lineage that have a large size and are nucleated. In zebrafish, a population of macrophages that originates from the ventro-lateral mesoderm and migrates to the YS, has been detected at the same time as the first erythrocytes emerge. These cells bypass the conventional developmental pathway that lead to macrophage differentiation in the hematopoietic organs (Herbomel et al., 1999). In the mouse, after the first nucleated erythrocytes there is, similarly to zebra fish, a wave of macrophage progenitors (2–4 somite stage S) shortly followed by the generation of erythro-myeloid precursors (4–6S) {Bertrand et al., 2005, #64628}. Lymphocyte progenitors are not consistently detected in the YS prior to the establishment of circulation (>5S). After circulation is established, it is unclear whether additional waves of hematopoietic generation occur *in situ* and whether they contribute to adult hematopoiesis. Although suspected for a long time, the function and fate of the early YS myeloid progenitors has been recently reassessed. It was shown that they migrate via the circulation to the central nervous system where they form a stable macrophage compartment, the microglia. Although a slow turnover rate of these cells is difficult to rule out, it appears that most adult microglial cells are of embryonic YS origin (Ginhoux et al., 2010). The function of the microglia is not totally elucidated, but they are known to contribute to the elimination of debris and dead cells during development, and also to inflammation in certain pathologic conditions. YS macrophages also contribute to epidermal specific macrophage pool called the Langerhans cells, as well as to liver Kupffer cells. Their independence from the expression of the transcription factor *c-myb*, suggests that these particular subsets of macrophages do not follow the developmental pathway taken by the progeny of HSCs (Schulz et al., 2012).

### **Intra-embryonic Hematopoietic Generation**

Experiments in several animal models, as previously mentioned, indicated that YS hematopoietic generation is independent from intra-embryonic generation and that there is no compelling evidence of its contribution to adult hematopoiesis. Isolating defined embryonic structures and analyzing their *in vivo* reconstitution capacity obtained the initial evidence that, in the mouse, progenitors of intra-embryonic origin were multipotent and capable of long-term reconstitution of the hematopoietic system. In one

experimental approach, the engraftment of splanchnic mesoderm, an embryonic structure close to the aorta anlage of 10–18S embryos, under the kidney capsule of severe combined immune deficient (SCID) mice, resulted in the reconstitution of the lymphoid compartment (Godin et al., 1993). Under the same conditions YS yielded no lymphocytes, indicating that only the intra-embryonic region contained HSCs. The absence of reconstitution with YS was interpreted as an indicator of an intra-embryonic origin of the reconstituting cells. Similar conclusions were drawn from other systems. Embryonic territories were isolated and cells injected in irradiated hosts to measure CFU-S activity. This experiment targeted embryonic structures isolated from embryos at later stages of development, after the splanchnic mesoderm had evolved into a territory containing the anlage of the aorta, the gonads and the mesonephros (AGM) (Medvinsky et al., 1993). A comparison between CFU-S obtained from YS, FL and AGM region showed that AGM was the first site to display CFU-S activity, followed by the YS and later the FL. These two experimental approaches relied on the precise identification and careful dissection of transient embryonic structures, at precise developmental times. The interest in the splanchnic mesoderm/AGM raised by these results rapidly spread in the scientific community. Shortly thereafter, it was shown that not only did AGM cells function as bona fide CFU-S, but also that they were capable of long term reconstitution (LTR) of the hematopoietic compartment, after E10 (Muller et al., 1994). LTR activity was detected in the YS and in the FL at later stages and followed similar kinetics to that observed for CFU-S. These results supported the idea that HSCs were generated in the intra-embryonic AGM, but do not provide direct evidence for this, as cells originated elsewhere could circulate to the aorta region. Direct evidence for an AGM origin of multipotent progenitors came from experiments where short periods of organ culture allowed the development of progenitors so that they became detectable in conventional hematopoietic assays. In this work the splanchnic mesoderm was dissected along with the YS and the remains of the embryo body from E7–E8.5, around the time where circulation and YS hematopoietic activity are established. After a few days in organ culture, multipotent progenitors were detected exclusively in the intra-embryonic territory, but not in YS or in the remaining embryo (Cumano et al., 1996). These results firmly established the splanchnic mesoderm/AGM origin of lympho-myeloid multipotent progenitors and their independence from YS. Cells within any of these explants were, however, incapable of reconstituting the hematopoietic system of normal hosts. Immune-deficient hosts proved useful to reveal HSC activity from tissues isolated early in embryonic development. *Rag2* (recombination activation gene 2) and *IL2rgc* knock-out mice (*Rag<sup>-/-</sup> IL2rgc<sup>-/-</sup>*) are double null mutants for the *Rag2* and for the interleukin 2 receptor gamma chain, and have neither T or B lymphocytes,

nor NK cell activity. When reconstituted with cells recovered from cultured explants of E8, long-term reconstitution was observed in hosts that received splanchnic mesoderm, but not YS (Cumano et al., 2001). The efficiency of reconstitution was, however, consistently lower than that obtained with E11 AGM. These results can be explained by at least two non-exclusive possibilities. Successive waves of generation result in the production of multipotent cells without reconstitution capacity and at a later time point, of HSCs that establish the hematopoietic system. Alternatively, there is a unique wave of hematopoietic progenitors that need further maturation to acquire reconstitution capacity, possibly through specific interactions with a specialized environment. The latter possibility is probably correct. Several lines of evidence suggest that hematopoietic progenitors are generated in the AGM in a very short time-window, followed by maturation and acquisition of properties of BM HSC. 1. The number of multipotent hematopoietic progenitors, present in the AGM across mid-gestation sharply increases between E9-E10, reaches a maximum at E10.5 and decreases thereafter, a result compatible with a single wave of hematopoietic cell production (Godin et al., 1999). 2. Although several lympho-myeloid progenitors are present in E11 AGM, the number of HSCs harboring LTR activity is one cell per AGM. This number is dramatically increased if *in vivo* transfer is preceded by culture for a few days, a result more compatible with *in vitro* acquisition of adult HSC properties (Rybtsov et al., 2011). 3. *In vivo* fate mapping studies of labeled cells expressing VE-Cadherin (Cdh5) (Zovein et al., 2008) or Runx1 (Samokhvalov et al., 2007), is found in endothelial and hematopoietic cells. These experiments show that induction of Cre recombinase, between E8.5 and E9.5, results in the expression of the reporter protein in virtually 100% of adult HSC. When induction is done before or after this short time-window, reporter expression in adult cells is either very low or undetectable.

These observations concur to the conclusion that mesoderm progenitors are specified into the hematopoietic lineage, once in a lifetime, between E8.5 and E9.5–10. Newly generated hematopoietic progenitors cannot reconstitute adult hosts, but must still acquire that capacity through a maturation phase that takes place in the AGM, and probably also elsewhere (FL or placenta). It should be pointed out that the requirements for FL or BM HSC colonization and differentiation may be distinct, because the FL is of endodermal origin, while the BM derives from mesoderm. In consequence, testing embryonic cells by their capacity to reconstitute adult organs might not be an optimal method to assay the properties of these cells *in vivo*.

The AGM is a complex territory comprising not only the dorsal aorta but also the anlage of the gonads and mesonephros. Newly generated hematopoietic progenitors are concentrated in the ventral aspect of the aorta, in cell formations protruding from the endothelial layer, which are



hematopoietic intra-aortic clusters (HIAC). The spatial confinement of the HIACs to the ventral aorta has been explained by finding in chicken embryos that the early endothelial layer of the ventral aorta derives from the lateral mesoderm (Pardanaud et al., 1989) which is replaced by conventional, somite-derived endothelial cells only after HSC generation has occurred (Pouget et al., 2006).

Similar to HIAC, cell aggregates are also found in the omphalo-mesenteric and vitelline arteries (de Bruijn et al., 2000; Garcia-Porrero et al., 1995; Godin et al., 1999), and it is therefore possible that this process also takes place in other major blood vessels connecting the embryo to the placenta and the YS.

### **Other Potential Hematogenic Sites—The Placenta**

In birds, the allantois is a diverticulum that projects in the extra-embryonic compartment and opens in the terminal part of the hind-gut. It is lined by endoderm and covered by mesoderm. Quail allantois grafted into chicken embryos resulted in partial BM colonization by donor derived hematopoietic cells, suggesting that allantois might also contribute to hematopoiesis (Caprioli et al., 1998). In mouse and man, the placenta has been suggested as a site providing a favorable environment for HSC maintenance and/or expansion (Gekas et al., 2005; Robin et al., 2009). It was shown that mouse mid-gestation placenta contains long-term reconstituting cells in numbers that parallel those found in the AGM. However, it has been difficult to determine whether the mammalian placenta could also be a site for HSC generation, as suggested (Gekas et al., 2005; Robin et al., 2009). HSC activity is detected in E9.5 placenta of mice that lack heart beat (*Ncx1<sup>-/-</sup>*) and where, therefore, cells should transit via circulation (Rhodes et al., 2008). These embryos, that die around E10, were analyzed at earlier time points (E8–E9.5) and found to contain lymphoid and myeloid progenitors in YS, AGM and placenta, although a lower number of hematopoietic cells were detected in mutant mice. This result raises a number of issues: 1. It is possible, as suggested by the authors, that placenta is a site of hematopoietic cell generation 2. Stem cell activity in these embryos was not directly assessed. so the actual generation of HSCs has not been addressed but only that of lymphoid and myeloid progenitors (Kieusseian and Cumano, 2008) 3. It has been well documented that the omphalo-mesenteric and vitelline arteries, similarly to the aorta, are a site for generation of multipotent hematopoietic progenitors (reference). These are major vessels that connect the embryo proper to the YS and to the placenta. The extent to which the generation process extends from the vessels into the territory that is isolated as YS or placenta, is unknown. It is therefore possible that some hematogenic

activity attributed to these extra-embryonic sites actually comes from the major arteries that are contained in the explant.

It should be pointed out that HSC generation connected to the endothelial wall of major vessels is a universal phenomenon that is observed in all species investigated so far (man, mouse, birds, fish and amphibian).

## THE GENERATION OF HEMATOPOIETIC STEM CELLS

It was clear from the experiments described above that although YS or placenta may contribute to adult hematopoiesis, all lines of evidence point to the splanchnopleura/AGM region as the major source of HSC. The characterization of the phenotype, the transcription factors involved, and the direct visualization of the generation process in the AGM then became the next goal.

### Phenotype

The first marker characteristic of embryonic HSC was identified when different subsets of FL cells were analyzed for LTR activity. The surface marker AA4.1 (CD93) was then identified as labeling a population that contained all HSCs (Jordan et al., 1990). In contrast to BM, FL HSCs are also CD34<sup>+</sup>. Apart from the expression of CD34 and AA4.1, FL HSCs are quite similar in phenotype to BM HSCs. Like BM they also express Kit and Sca1 in the Lin<sup>-</sup> compartment, and CD150 in the CD48<sup>-</sup> subset.

The first attempts to characterize the progenitor compartment in AGM indicated that AA4.1 was also expressed by AGM progenitors while Sca-1 was undetectable (Godin et al., 1995). Like in FL, Kit and CD34 are markers of AGM repopulating cells (Sanchez et al., 1996). *In situ* labeling of cells in the HIACs showed that another marker, CD41, that is only present in BM megakaryocytes, also marks AGM multipotent progenitors. Thus Kit and CD41 are the phenotypic markers that identify the first hematopoietic cells. These cells also co-express CD31, CD34, AA4.1, VE-Cadherin and Tie-2, all of which are present on endothelial cells (Bertrand et al., 2005a), and they do not have detectable levels of the pan-hematopoietic marker CD45. This led to the notion that both lineages were developmentally related. From the analysis of the hematopoietic activity of sorted cells, several features can be highlighted: 1. Strict hematopoietic (Kit and CD41) and endothelial/hematopoietic markers (CD34, CD31, VE-Cadherin, Tie-2) are co-expressed by cells with a unique hematopoietic potential, even in the absence of CD45. This pattern of expression created some confusion because incomplete analysis led to the wrong affiliation of hematopoietic cells to the endothelial lineage. 2. By E11 most hematopoietic cells express CD45

that is progressively acquired in Kit<sup>+</sup> cells 3. The set of markers mentioned here labels most colony forming cells, irrespective of their multipotent nature and of their origin, so that erythro-myeloid YS cells that cannot generate lymphocytes express the same set of markers as AGM-derived reconstituting cells.

## **HSC Generation is Conditioned by their Transcriptional Profile**

Several transcription factors are essential for the establishment of a functional hematopoietic system. Factors required for erythrocyte formation are expressed during YS development and their absence results in early embryonic lethality. Without the availability of conditional knock-out mice their specific roles in HSC generation remain unclear. This is the case for Tal1/Scl, Lmo2 and Gata1. Mutations in cytokine receptors that modulate endothelial cell development will also result in early embryonic death with abnormal YS formation. The receptors Flk1 (Kdr, Vegf receptor 2) and Tie-2 (angiopoietin receptor) belong to this category. Inactivation of other transcription factors and cytokine receptors spare YS erythropoiesis allowing the embryonic development to progress to stages where HSC are detected. This is the case of AML1/Runx1 and Cbfb (core binding factor beta, partner of Runx1), Gata2, Myb (c-myb), Pu.1 (Sfpi1) and Ikaros (Ikzf1). We do not pretend to be exhaustive in this description; rather, we have concentrated on a few well-documented examples. Runx1 is expressed in hematopoietic, endothelial and mesenchymal components in mid-gestation mouse embryos. It was shown that Runx1 is essential for HSC generation because deletion in VE-Cadherin expressing cells results in an absence of HSC. In contrast, deletion in cells expressing *Vav*, which occurs at later developmental stages, has no detectable functional consequences (Chen et al., 2009). Because VE-Cadherin is expressed on hematopoietic and endothelial cells, it was also postulated that Runx1 absence affected HSC differentiation from the hematogenic endothelium. It was recently shown that Cbfb expression was required for erythro-myeloid cells and HSC generation. However, expression of Cbfb driven by Tie-2 regulatory sequences was sufficient for erythro-myeloid but not for HSC formation, only detected when expression was driven by a Ly6a promotor (Chen et al., 2011). These data are interpreted as evidence for the existence of different progenitors originating erythro-myeloid cells and HSC. Overall the experiments where transcription factors and cytokine receptors were inactivated have shown the potential requirement of these factors in hematopoietic generation, but the underlying mechanisms have not been systematically addressed and are likely to be part of a complex cascade of events.

## Visualization of the generation process

The coincidence of surface markers and transcriptional requirements in hematopoietic and endothelial cells led to the general notion that hematopoietic cells derive from endothelial compartments, and the site of origin is therefore called hematogenic endothelium. Although this notion derived from the proximity between the two cell-types, a real-time documentation of the sequence of events leading to HSC generation would bring new information on the details of the process.

Several attempts have been made to record hematopoietic generation from differentiating mammalian ES cells. It was shown that adherent cells, with the morphology and surface expression pattern of endothelial cells, could generate hematopoietic cells at the clonal level (Choi et al., 1998; Eilken et al., 2009). Experiments with ES cells, however, raise the question of the stage of differentiation of the cell that initiated the colony, a matter always difficult to assess precisely. The trivial possibility that a mesoderm progenitor differentiates into two out of the many possible tissues has not been ruled out. It should also be stressed that although erythroid differentiation is frequent in ES cell cultures, and lymphocytes can be observed under particular culture conditions, HSCs with the capacity to contribute to a host hematopoietic system *in vivo* were never obtained.

While real-time visual documentation of developmental processes in mammalian embryos is difficult, the Zebra fish model is highly appropriate for this requirement, due to the transparency of the embryo and the availability of a large collection of reporter-expressing strains. The analysis of fish embryos showed that cells with the morphology, expression pattern, and spatial localization of endothelial cells could adopt the morphology of hematopoietic cells. The process is highly polarized, occurs without cell division and requires Runx1 expression. The hematopoietic cells immediately migrate into the sub-aortic space, but not in the lumen. This generation process, which does not occur through asymmetric cell division, has been called endothelial to hematopoietic transition (Bertrand et al., 2010; Kissa and Herbomel, 2010). In the mouse embryo, the process has been more difficult to visualize in real time by *in situ* imaging (Boisset et al., 2010), but immunohistochemical analysis showed that a similar process might occur in mammalian embryos (Bertrand et al., 2005a; Ivanovs et al., 2011; Tavian et al., 1996; Yokomizo and Dzierzak, 2010). Some differences however should be noticed. The images available in the chicken, mouse, and human suggested that HSCs were present in HIAC, as aggregates initially attached to the endothelial lining of the ventral aspect of the dorsal aorta, and were subsequently released in circulation (Bertrand et al., 2005a; Ivanovs et al., 2011; Tavian et al., 1996; Yokomizo and Dzierzak, 2010). The presence of a few distinct cell aggregates that were documented in many different

species (except Zebra-fish) suggests that either very few cells initiate the hematopoietic transition and expand thereafter, implicating a clonal nature of HIACs, or that transition occurs in defined regions of the aorta where a number of cells initiate the process and stay aggregated. In Zebra fish, individual and newly differentiated cells migrate to the sub-endothelial space; structures similar to HIACs have not been observed.

While most newly generated hematopoietic cells are found within the HIAC, in the luminal side of the aorta and major vessels, some observations in the mouse model showed a few of these cells in the sub-aortic region (Manaia et al., 2000). This led to the suggestion that rather than having an endothelial origin, hematopoietic progenitors derive from already committed cells that cross the endothelial lining and transiently assume an endothelial position (Bertrand et al., 2005a). Some experimental evidence indeed supports the notion that the sub-endothelial hematopoietic cells are more immature than those found in the HIACs (Rybtsov et al., 2011). While this remains a possibility that is compatible with the strict hematopoietic potential of hematogenic endothelium, there is an underlying timing incoherence. If we accept that lineage tracing experiments timed hematopoietic generation between E8.5–E9.5–10, it is unlikely that sub-aortic cells, which are only detected after E10, are the progenitors of the HIAC cells that appeared earlier. By E10.5, the generation process is completed and the sub-aortic progenitors could simply be a few newly generated cells that, rather than protruding in the lumen, go the opposite way, similarly to what was observed in Zebra fish. Whether these cells contribute to adult hematopoiesis, is unknown. Alternatively, it is possible that they correspond to the last few progenitors that cross the endothelial layer at the end of the generation process. The functional analysis of cells corresponding to specific spatial locations should resolve that controversy.

After being generated in the splanchnopleura/AGM, cells are found in circulation and shortly after in the FL, the major embryonic hematopoietic site (Delassus and Cumano, 1996). Somewhat later (E10.5–11), the thymus anlage is colonized and becomes the only site for T cell production that will be active throughout life. While mature blood cells differentiate in the FL and thymus, the placenta may be a site where HSCs transiently home (Gekas et al., 2005).

Hematopoietic embryonic development follows a common general pattern observed in all vertebrate species studied that include fish, amphibian, avian, mouse, and human embryos. It starts with the initial generation in the YS (ventral blood islands for *Xenopus* and ventral lateral mesoderm in Zebra fish) of erythrocytes and some myeloid progenitors, in the apparent absence of HSCs. These primitive cells that differentiate *in situ*, contribute transiently to erythro-myelopoiesis. A second generation, of multipotent progenitors and HSCs in the splanchnic mesoderm/AGM,

follows. These cells are generated in low numbers and for a limited period of time, establishing the hematopoietic system for life. HSCs or their precursors migrate to an intermediate hematopoietic organ, the FL in mammals and amphibian, the para-aortic foci in avian and the caudal hematopoietic tissue (Murayama et al., 2006) in Zebra fish. In this intermediate site HSCs differentiate, but more importantly, expand. Finally, cells migrate to the definitive hematopoietic organ where production of all blood cells (with the exception of T lymphocytes) occurs throughout life, the BM in mammals and birds (which also produce blood cells in adult spleen) and the kidney in fish and amphibians. In mammals, the spleen is a secondary lymphoid organ in adults and a transient hematopoietic organ during fetal life that, in contrast to FL, is specialized in the production of only a few cell types (Bertrand et al., 2006).

## **THE FETAL LIVER**

The liver anlage develops from endodermal derived progenitors that invade the mesenchyme around the septum transverse, between the 7 and the 11-somite stage. Cells from the endoderm adopt a columnar morphology and express several hepatic genes comprising albumin, alpha-fetoprotein and hepatocyte nuclear factor 4a (Hnf4a) that are good indicators of the hepatic cell fate (Si-Tayeb et al., 2010). In mammals, soon after the liver progenitors invade the surrounding mesenchyme, the FL is colonized by hematopoietic progenitors and transiently becomes the principal hematopoietic organ. Co-culture studies have suggested that immature hepatic progenitor cells can generate an environment that supports hematopoiesis (Hata et al., 1993); however, when hepatic progenitor cells are induced to differentiate to a mature form, the resulting cells can no longer support blood cell development (Kinoshita et al., 1999), consistent with the movement of hematopoietic stem cells from the fetal liver to the adult bone marrow, during this general time frame. In addition to the parenchymal cells, fetal liver-derived stromal cells have been shown to enhance hematopoietic progenitor cell proliferation possibly through Wnt signaling pathways (Martin and Bhatia, 2005).

Colonization of the FL by AGM-derived blood-borne HSCs starts on E 10, in a process that requires expression of b1 integrin by the colonizing HSCs (Potocnik et al., 2000). In the FL environment HSCs expand and differentiate into multiple hematopoietic lineages. This organ then becomes the major embryonic site where blood cells are generated throughout gestation. Starting on day 15 of embryonic life hematopoietic precursors also exit the FL to colonize the inner cavities of bones where increasing hematopoietic activity is detectable until the end of gestation. Shortly after birth, this process—again dependent on the expression of b1 integrin by the

precursor cells (Potocnik et al., 2000)—is completed and the bone marrow takes over as the primary hematopoietic site, throughout adult life.

The first cell type detected after the onset of FL hematopoiesis is mature (enucleated) erythrocytes. The rapidity with which mature erythrocytes become the predominant form detected in the fetal blood (over 80% by day E 12) is probably due to rapid maturation of nucleated yolk sac-derived erythrocytes that become exposed to erythropoietin in the liver. This indicates that not only AGM-derived HSCs, but also YS progenitors can enter the fetal liver and eventually complete maturation in this organ.

The first committed cells that developed in the FL from HSCs are detected only four days after the onset of liver colonization. Cells of the myeloid lineage can be detected as early as day 14, concomitantly with the first CD19<sup>+</sup> B-lineage cells. However, only by day 17 are IgM-expressing B lymphocytes detected. Since YS progenitors do not have lymphocyte precursor potential, these B lineage cells are the first that necessarily must have resulted from differentiation of HSCs in the fetal liver. The short time (seven days) elapsed between the detection of mature hematopoietic cells and the colonization of the FL indicates that HSC differentiation start immediately after migration of HSCs into the liver.

## **Hematopoietic Stem Cells in the FL**

HSCs colonize the FL starting at E10–E11. By the time they reach this organ, HSCs express Kit and AA4.1 (Jordan et al., 1990) but have low levels of Sca1 that is difficult to detect by antibody staining. However, in Sca1-GFP transgenic embryos, HSCs express GFP already in the AGM (de Bruijn et al., 2002). At this early stage, they also do not express CD150 and most cells are CD48<sup>+</sup>. Full maturation to an HSC adult phenotype occurs in the FL by the acquisition of Sca1 and CD150 expression that is completed between E12 and E13 (Kim et al., 2006).

Two major differences distinguish FL and adult BM hematopoiesis. On the one hand, differentiation of HSCs in the FL proceeds as a synchronous wave of differentiation (Ema and Nakauchi, 2000) with a defined start point (at day 10–11, after colonization), and therefore throughout embryonic life, the FL is enriched in early precursors, with a relatively poor representation of mature populations. This contrasts with the situation in the BM where ongoing hematopoiesis results in a predominance of more mature cell types and a low representation of early progenitors.

On the other hand, the FL environment provides the developing cells with signals distinct from the ones they encounter in the BM and, in contrast to adult HSCs, the ones in FL proliferate and increase in numbers (Kim et al., 2006). Long-term reconstitution of lethal irradiated recipients is obtained starting at E11–E12 and the numbers of HSCs increase with a doubling

time of 24h up to E16. It was calculated that HSCs increase up to 40 times, in FL, until E16 and decrease thereafter (Ema and Nakauchi, 2000). The progressively reduced number of HSCs in the FL after E16 is concomitant with the colonization of the BM. Whether the exit of HSCs from FL is due to gradual impairment of the stromal compartment, active recruitment to the BM, or to both, or other mechanisms remains to be elucidated.

## **The Lymphoid Compartment**

The different signals provided in the two environments are likely the main reason for the well known distinct properties displayed by precursors at the two sites. Fetal precursors, developing in a fetal environment, are the only source of the Vg3 expressing gamma/delta T cells (Tgd) (Garman et al., 1986; Ikuta et al., 1990). Fetal B cell progenitors are particularly efficient at generating the B1 and MZ compartments (Barber et al., 2011; Herzenberg, 1989).

In addition, we have found that pro-B cells isolated from the FL are unique in their ability to proliferate in response to the cytokine Tslp (thymic stromal lymphopoietin), while bone marrow derived pro-B cells are strictly dependent on IL7 (Carvalho et al., 2001; Vosshenrich et al., 2003). Two observations support the notion that this differential response to Tslp is due to the fact that pro-B cells in the FL are subject to different environmental signals: in mice at one week of age, pro-B cells from the bone marrow have a much lower frequency of response to Tslp, compared to the response of the few pro-B cells that can still be isolated from the liver of the same animals (Vosshenrich et al., 2004); and bone marrow pro-B cells acquire response to Tslp when used to reconstitute FL explants in a fetal liver organ culture (unpublished).

Genome wide microRNA analysis of precursors from fetal liver or bone marrow origin reveals a substantially different transcriptome in pro-B cells from the two sites (unpublished. See also IMMGEN-The Immunological Genome Project—at: [http://www.immgen.org/index\\_content.html](http://www.immgen.org/index_content.html)). It was recently reported that Lin28b (Lin-28 homolog B), a repressor of the let7 family of miRNA, is highly expressed in fetal but not in adult hematopoietic precursors (Yuan et al., 2012). Remarkably, ectopic expression of Lin28b in adult precursors confers them with properties characteristic of fetal progenitors, such as expression of elevated levels of *Hmga2* and *Igf2*, partial IL7 independency of B cell development, high efficiency in the reconstitution of the B1 and MZ compartments, and generation of a large CD4<sup>+</sup>Vg1.1<sup>+</sup>Vd6.3<sup>+</sup> gamma delta T cell population, that is only produced in perinatal life (Yuan et al., 2012).



This finding indicates that Lin28b control of miRNA expression is a regulator responsible for the different properties displayed by fetal or adult precursors. An unresolved question is to which extent external signals are responsible for Lin28b expression in fetal life. It is likely that differences in the stromal components in FL or BM imprint the stem cells with characteristic “fetal-like” or “adult-like” mRNA signatures. However, not enough information is available regarding hematopoietic stromal cells, their identification, the factors they produce or the signals they deliver to define the mechanisms involved.

## **THE FETAL SPLEEN**

The spleen is an organ located in the posterior aspect of the stomach, in close contact with the omentum and the pancreas. While the pancreas originates from a mesodermic induction of the endoderm (Edlund, 2002), the spleen is considered of exclusive mesoderm origin (Thiel and Downey, 1921). At E12.5, the spleen starts to be distinguished from the pancreatic anlage and already contains multiple hematopoietic progenitors. The spleen displays hematopoietic capacity during the embryonic and neonatal period and then mainly becomes a secondary lymphoid organ, in mammals.

### **The Hematopoietic Stem Cells in the Fetal Spleen**

Long-term reconstitution assays detected HSCs in fetal spleen (FS) between E13.5 and E18.5 (Godin et al., 1999). Circulating HSCs constantly colonize the spleen throughout fetal development (Bertrand et al., 2006; Christensen et al., 2004). Splenic HSCs are rare and difficult to detect at early fetal stages (Bertrand et al., 2006), but are phenotypically and functionally identical to their FL counterparts (Kiel et al., 2005). Considering the number of reconstitution assays and the frequency of the FS HSC engraftment, it was calculated that less than five HSCs are present in the spleen, increasing in number to reach more than 100 per spleen around E17.5 (GE Desanti and R Golub, unpublished). The increase in HSC number in the FS during the late gestational period parallels the increased colonization of the BM as hematopoiesis in the FL decreases (Christensen et al., 2004). During this transition, the FS hosts part of the circulating HSCs released from the FL while thereafter, they are found as barely detectable fractions in the adult spleen as well as in other extra-medullary sites (Massberg et al., 2007).

Contrary to FL the FS environment is unable to maintain an HSC pool, indicating that specific niches for their maintenance are not found in the FS (Bertrand et al., 2006). HSC could only generate myeloid progeny in the

splenic environment, suggesting that FS macrophages may derive in part from the endogenous splenic HSC pool.

## The Hematopoietic Progenitors in the Fetal Spleen

### *The erythroid subset*

From E12 to birth, the FL is the main site for erythropoiesis, while the BM produces the blood cells during the neonatal and adult period. In E14.5 FS, erythroid Ter119<sup>+</sup> cells are predominant compared to CD45<sup>+</sup> leukocytes. At E15.5, clear erythroid populations are distinguished from immature proerythroblasts (Ter119<sup>int</sup>CD71<sup>hi</sup>), basophilic erythroblasts (Ter119<sup>hi</sup>CD71<sup>hi</sup>), late basophilic and polychromatophilic erythroblasts (Ter119<sup>hi</sup>CD71<sup>int</sup>) and mature orthochromatophilic erythroblasts (Ter119<sup>hi</sup>CD71<sup>-/lo</sup>) (Desanti et al., 2007). The increase in the frequency of late erythroid precursors between E13.5 and E15.5 supports the idea that erythropoiesis also occurs in the FS during this period. In the adult, very few early (Lin<sup>-</sup>Kit<sup>+</sup>CD71<sup>-</sup>Gata1<sup>+</sup>) and late (Lin<sup>-</sup>Kit<sup>+</sup>CD71<sup>+</sup>Gata1<sup>+</sup>) erythroid progenitors are found in the spleen (Suzuki et al., 2003). After phenylhydrazine-induced hemolytic anemia, the proportion of these progenitors increases, indicating that the spleen can be an extra-medullary erythropoietic site in anemic conditions (Migliaccio and Migliaccio, 1998; Suzuki et al., 2003).

### *The myeloid subset*

The macrophages sequentially found in the spleen have different origins. At E12, spleen macrophages can be detected by the expression of the F4/80 antigen (Morris et al., 1991). These macrophages are found before the initiation of intra-embryonic hematopoiesis, supporting the idea that they originate in the YS, the main source of mature macrophages at this stage (Bertrand et al., 2005b). Macrophages are later generated *in situ* derived from resident progenitors. At E15.5, the FS contains around 40% of F4/80<sup>+</sup>Mac-1(CD11b)<sup>+</sup> macrophages. The phenotype of splenic macrophages changes between embryogenesis and neonatal life. Indeed, all splenic macrophages are F4/80<sup>+</sup>Mac-1<sup>+</sup> at E15.5, but lose Mac-1 expression after birth, thus acquiring the phenotype of adult red pulp macrophages. We propose that FS hematopoiesis gives rise to the first neonatal red pulp macrophages.

Dendritic cells (DCs) are major players in the establishment of the immune function of the spleen, since they initiate T-cell dependent immunity. So far, splenic DC development during fetal life has not been investigated. Two groups have described that splenic DCs are identified at day 1 after birth with a prominent CD4<sup>-</sup>CD8<sup>-</sup>CD11c<sup>+</sup> DC subset that is poorly

represented in the adult spleen (Dakic et al., 2004). During the second week of life, other DC subsets are progressively detected in the spleen (CD4<sup>-</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>-</sup> DC populations). The double-negative subset, that constitute the majority of neonatal DCs, has been shown to exhibit similar properties to the adult CD8<sup>+</sup> DC subset, and could be considered the neonatal precursor of these cells (Dakic et al., 2004). The splenic stroma has been shown to be involved in the differentiation and maturation of DCs (Ni and O'Neill, 1999). Neonatal splenic stromal cells could provide a suitable environment for the maturation of DCs into regulatory DC, which can modulate the immune response in the spleen (Zhang et al., 2004).

### ***The lymphoid subset***

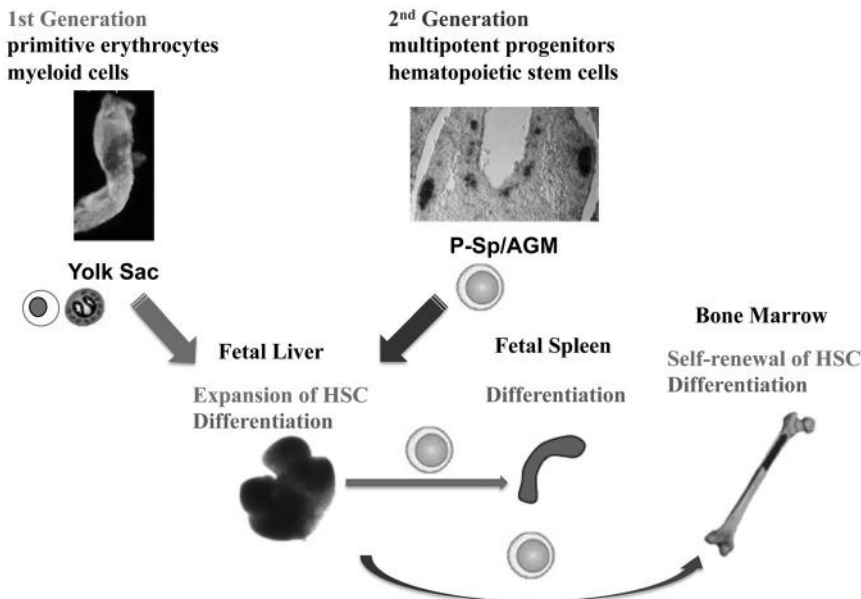
The FS is colonized by erythroid, myeloid and lymphoid progenitors as early as E12.5–E13 (Desanti et al., 2008; Godin et al., 1999). Between E14.5 and E15.5, FS hematopoietic cells lack lineage specific markers (Lin<sup>-</sup>). As a future secondary lymphoid organ, the FS contains the lymphoid subset of lymphoid tissue inducer (LTi) cells. These cells can be found in the anlagen of all future secondary organs (Eberl and Littman, 2004; Mebius et al., 2001; Yoshida et al., 1999). FS LTi cells are mainly found in a peri-arteriolar position, where chemokines can be detected in stromal and endothelial cells. These cells are located in the white pulp anlage (Vondenhoff et al., 2008). The LTi cells originate from FL progenitors that migrate via the mesenteric vessels between E11.5 and E15.5 to colonize the gut, the spleen and LN (Mebius et al., 2001; Possot et al., 2011; Yoshida et al., 2001). To assess the *in situ* differentiation of splenic Lin<sup>-</sup> progenitors, we performed E15.5 FS explant organ culture (FSOC). Reconstitution assays of FSOC by progenitors, as well as cultures of these progenitors on fetal spleen stromal cells, showed that they could differentiate into B and NK lymphocytes and myeloid cells (Desanti et al., 2008). B cell progenitors were detected in the Lin<sup>-</sup>CD4<sup>int</sup>Rag2<sup>hi</sup> subset at E14.5 and E15.5 (Desanti et al., 2008). Moreover, a low percentage of B cells (CD19<sup>+</sup>B220<sup>+</sup>Rag2<sup>+</sup>) was already observed at E13.5 (Bertrand et al., 2006). Thus, in this environment, B lymphopoiesis is supported in this environment and the first IgM<sup>+</sup> cells are detected in E17.5 FS, at the same time as in FL (Bertrand et al., 2006; Velardi and Cooper, 1984a, b). In two week-old neonates the lymphopoietic activity of the spleen is interrupted and the spleen only supports B cell maturation. In osteopetrotic mice (*op/op* and *mi/mi* mice) with extra-medullary hematopoiesis, it was shown that, contrary to the liver, the adult spleen was devoid of B cell progenitors (Tagaya et al., 2000). *IL-7*, *Flt3L* and *Cxcl12* transcripts have been detected in the spleen of these mice, suggesting the expression of cytokines and chemokines involved in B cell lymphopoiesis. The reason why the spleen switches from primary to secondary lymphoid organ after birth is still

unclear. Nevertheless, the capacity to sustain myeloid development is maintained in the adult spleen.

## The Adult Spleen as an Alternative Site of Hematopoietic Development in Particular Situations

The adult spleen is known for its capacity to store platelets, remove senescent erythrocytes via the F4/80<sup>+</sup> phagocytic red pulp macrophages, and also to support B cell maturation (Allman et al., 2001; Brendolan et al., 2007a; Brendolan et al., 2007b; Cancro et al., 2001; Shimizu and Hokano, 1988). This organ also hosts BM-derived basophil/mast cell precursors that increase in number upon IL3 stimulation (Arinobu et al., 2005; Ohmori et al., 2009). The spleen is a unique organ with an MZ structure where MZ B cells are observed; the lack of these cells is associated with impaired clearance of blood borne bacterial pathogens and the inability to mount a rapid immune response against streptococcal polysaccharides.

In particular situations, such as hematopoietic stress and myeloproliferative disorders, the spleen is able to sustain an important erythro/myeloid differentiation program, referred to as extra-medullary hematopoiesis.



**Figure 1.** The developmental pathway of hematopoietic stem cells. The diagram shows the major steps of fetal hematopoiesis. Nucleated primitive erythrocytes; myeloid cells; stem and progenitor hematopoietic cells.

After BM cell mobilization by cyclophosphamide/G-CSF treatment or intravenous reconstitution of irradiated mice, HSCs can be found in the parafollicular red pulp of the spleen, in close contact with the sinusoidal endothelium (Kiel et al., 2005). Splenic sinusoidal endothelial cells express CXCL12 and thus can attract hematopoietic progenitors (Sugiyama et al., 2006). Splenic colonies after HSC transplantation become a major site of megakaryopoiesis (Slayton et al., 2002) and after total BM reconstitution, CFU-S also harbors T cell progenitors (Arcangeli et al., 2005; Lancrin et al., 2002). Using a hypomorphic *Gata1* mutant mouse, where hematopoietic cells generated in the BM are unable to express Gata1, it was shown that the extra-medullary environment of the spleen could support differentiation and maturation of HSCs (Ghinassi et al., 2010; Migliaccio and Migliaccio, 1998). In two transgenic mouse models that overexpress IL-5, the deficiency of hematopoietic activity in the BM is accompanied by extra-medullary hematopoiesis in the spleen, associated with multifocal ectopic bone formation, probably due to the migration of mesenchymal cells from the BM (Khaldoynidi et al., 2003; Macias et al., 2001).

The size of the BM cavity is linked to the extra-medullary activity of the spleen. *Cbfa1*<sup>-/-</sup> (*Runx2*<sup>-/-</sup>) mice completely lack both intramembranous and endochondral bone formation owing to the arrest of osteoblast maturation, resulting in a total lack of BM throughout the entire skeleton (Deguchi et al., 1999). E18.5 *Cbfa1*<sup>-/-</sup> embryos have splenomegaly and an increased number of splenic myeloid progenitors. Similar observations have been made in the osteopetrotic *op/op* mouse that present a transiently reduced bone marrow cavity due to the absence of M-CSF that results in osteoclast deficiency. Before four weeks of age, the absence of osteoclast activity does not influence the BM structure and the spleen has a normal number of cells and progenitors (Begg and Bertoncello, 1993; Nilsson et al., 1995). After that time, the reduced BM cavity leads to an increase in splenic hematopoietic progenitor activity and splenomegaly. With age, the femoral cavity progressively enlarges with consequent recovery of BM hematopoiesis and reversal of extra-medullary hematopoietic activity (Begg and Bertoncello, 1993; Nilsson et al., 1995). Two other mouse models with an important extra-medullary hematopoiesis in the spleen are *RANKL*<sup>-/-</sup> (*Trance*<sup>-/-</sup>, *Tnfs11*<sup>-/-</sup>) and *Fos*<sup>-/-</sup> (Miyamoto et al., 2011).

## CONCLUSION

The establishment of *in vivo* and *in vitro* assays that demonstrate multipotency and self-renewal as the fundamental properties of hematopoietic stem cells set the foundations of hematopoiesis as a scientific discipline and of modern stem cell biology. Close to fifty years have gone since the first CFU-S and long-term reconstitution assays were used to test the functional properties

of progenitor populations, but they are still used as the only tests available to probe stem cell activity. The development of flow cytometry and the availability of antibodies recognizing multiple cell surface determinants were instrumental in defining and isolating discrete progenitor compartments. This allowed the unraveling of the hierarchy of intermediate stages of differentiation that result in mature blood cells. Gene expression profiling and amplification strategies revealed signatures characteristic of differentiating cells and how the several transcription factors involved in the control of hematopoiesis affect the progenitor compartments. Many questions, however, remain elusive. Despite the impressive advances in molecular biology and sophisticated analysis of single cells, we still do not understand the molecular mechanisms determining hematopoietic stem cell specification from their mesoderm progenitors. As a consequence, protocols to derive large numbers of HSCs from ES or iPS cells have not been successfully established. Importantly, the signals that determine self-renewal rather than engagement into particular differentiation pathways are not understood at all and therefore we do not know the conditions to expand or maintain HSCs *ex vivo*. Dissecting the developmental processes that lead to the establishment of a functional hematopoietic system during embryogenesis will provide useful information to devise novel strategies to use adult tissue stem cells in regenerative medicine.

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## Abbreviations

|       |                            |
|-------|----------------------------|
| HSC   | Hematopoietic stem cells   |
| FL    | Fetal liver                |
| FS    | Fetal spleen               |
| BM    | Bone marrow                |
| CFU-S | Colony forming unit-spleen |
| LTR   | Long-term reconstitution   |
| E     | Embryonic day              |
| DC    | Dendritic cells            |
| AGM   | Aorta Gonads Mesonephros   |
| YS    | Yolk Sac                   |

|       |                                       |
|-------|---------------------------------------|
| S     | somites                               |
| SCID  | Sever combined immune deficiency      |
| IL    | Interleukin                           |
| HIAC  | Intra aortic hematopoietic clusters   |
| ES    | Embryonic stem                        |
| iPS   | Induced pluripotent stem              |
| Ig    | Immunoglobulin                        |
| GFP   | Green fluorescence protein            |
| MZ    | Marginal zone                         |
| TSLP  | Thymic stroma-derived lymphopoietin   |
| FLOC  | Fetal liver organ culture             |
| FSOC  | Fetal spleen organ culture            |
| G-CSF | Granulocyte colony-stimulating-factor |
| M-CSF | Macrophage colony-stimulating-factor  |

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# Neural Progenitors and Evolution of Mammalian Neocortex

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## SUMMARY

The mammalian neocortex develops from a population of primary neural stem cells called neuroepithelial cells. These cells, with the progression of development, differentiate into multiple populations of neural progenitors, which give rise to the neurons of the neocortex. These neural progenitors differ in their cell biological features and self-renewing ability, which in turn results in vast differences in the neuronal output. The differences in neuron number influence the gross morphology of the brain and the patterns of connectivity, which are very variable among different mammalian species. Work on mammalian neurogenesis has provided us with insight into the molecular mechanisms by which the neural progenitors self-renew and divide. The divisions of different neural progenitor populations are

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*List of abbreviations after the text.*

regulated by intrinsic, cell-autonomous factors, but also by signals coming from the surrounding tissues and other neural progenitors, all working in synchrony to tightly control the balance between proliferation and differentiation.

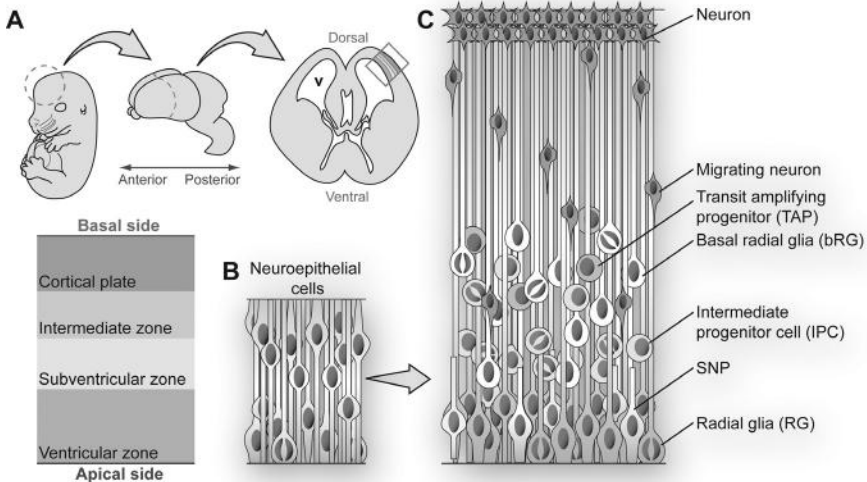
## **INTRODUCTION**

Mammals as a class are characterized by the presence of hair and mammary glands, but there is another feature that sets them apart from other animal classes—the relative increase in their brain size, especially its evolutionarily youngest region, the neocortex. The neocortex of an adult individual is a complex, postmitotic laminated structure, which is composed of six layers of neurons (as already noticed in the 19th century by Ramon y Cajal). The neurons in these layers differ in their cell biological features and the patterns of connections they make, and are responsible for the higher cognitive and associative processes that characterize mammals. Although vastly different in their positions and roles in the adult brain, almost all of these neurons are born from only a small number of progenitor types present in the developing brain. It is the subtle differences in cell biological features, modes of division, number of divisions, and cell cycle durations of these progenitors that give rise to this variety of postmitotic neurons.

Although neurogenesis as a process is quite conserved between different species, there are nonetheless important differences in progenitor cell composition which lead to the diversity of neurons and, ultimately, to the variation in connectivity and gross morphology of the brain of different mammalian species. Since the model organism mostly used for studying neurogenesis is the mouse, most of the references given in this chapter refer to murine neurogenesis. In the section concerning evolution of the brain, we will give examples of studies of neurogenesis in less frequently used model organisms and the important findings that have arisen from them. With this comparative approach we would like to stress the significance of this, unfortunately somewhat neglected, but recently again recognised strategy, in deciphering the processes underlying the development and adaptability of the mammalian brain.

## **NEURAL STEM AND PROGENITOR CELLS IN THE EMBRYONIC BRAIN**

The mammalian embryonic brain starts developing as a sequence of vesicles which arise from the neural tube (Figure 1A). Initially, the neural tube is comprised of neuroepithelial cells. These neuroepithelial cells will give rise to different types of stem and progenitor cells before and during the



**Figure 1. Development of the mammalian neocortex.** (A) Schematic of the mouse E14 embryo and its brain. Dashed line in the brain (middle) represents the position of the coronal section (right). The violet rectangle marked on the coronal section is shown in greater detail in C. This image shows different zones in the developing neocortex: germinal zones (violet and yellow), intermediate zone and the cortical plate where the newborn neurons settle. V=ventricle. (B) Scheme of the developing brain of an undefined mammalian species at a cellular level. Left—developing neocortex at an early stage, when the only cells comprising the cortical wall are NE cells. (C) Developing neocortex at an advanced stage. Different neural progenitor populations known until now are shown in various colours.

*Color image of this figure appears in the color plate section at the end of the book.*

process of neurogenesis. We will use the term **neural progenitors** to refer to all the populations of stem and progenitor cells that arise in the mammalian embryonic neocortex.

## Neuroepithelial Cells

Before the onset of cortical neurogenesis (before embryonic day (E) 10 in the mouse) the only cells comprising the neuroepithelium are the neuroepithelial (NE) cells (Figure 1B). The NE layer has a pseudostratified appearance (i.e., appears layered) although only a single layer is present, due to a process called **interkinetic nuclear migration (INM)** (see below). These cells divide rapidly, thus expanding the progenitor pool, from which other neural progenitors, and eventually neurons, will be born. At around E10, these cells start to show features of glial cells (Campbell and Götz, 2002; Kriegstein and Alvarez-Buylla, 2009).



## Radial Glia

The transition of NE to another cell type is accompanied by the appearance of glycogen granules (Gadisieux and Evrard, 1985) and the start of expression of some glia-specific markers, such as glutamate aspartate transporter (GLAST) (Shibata et al., 1997) and brain lipid-binding protein (BLBP) (Feng et al., 1994). Accompanying the changes in protein and intermediate filament expression, remodeling of cell shape takes place, with the thickening of the neuroepithelium and lengthening of neural progenitors occurring at the same time (Sidman and Rakic, 1973). This remodeling is paralleled by the expression of intermediate filaments like nestin (Hartfuss et al., 2001) and vimentin (Noctor et al., 2002). Although the cortical wall thickens significantly (up to a few millimeters in humans), the NE cells do not lose the apical and basal contacts, thereby remaining polarized. The type of protein complexes on the apical side changes, with the loss of neuroepithelial tight junctions but maintenance of adherens junctions (Aaku-Saraste et al., 1996). The elongated appearance and the glial nature of these cells are the reasons why these cells are referred to as **radial glia** (RG) (Figure 1C).

RG cells are the basic population of neural progenitors that will give rise either to neurons, through direct neurogenesis, or to other neural progenitor types. Their common features (in addition to previously mentioned glial markers and elongated shape) include the expression of the transcription factor Pax6 (see below Götz et al., 1998; Heins et al., 2002), transmembrane protein prominin-1 (CD133) (Weigmann et al., 1997) and mitoses at the apical surface. The latter feature is shared with NE cells, which is why NE and RG cells are collectively referred to as **apical progenitors** (APs) (Table 1). The restriction of mitoses to the apical surface is related to the process of INM and has implications regarding the modes of cell divisions (Taverna and Huttner, 2010). The agglomeration of RG nuclei in the area just above the apical surface creates a separate “zone” in the developing

**Table 1. Characteristics of neural progenitor populations.** A summary of cell-biological features of neural progenitor populations (see text). Transcription factors marked in grey indicate that this protein is present in the particular progenitor populations, but either at low levels (e.g., Pax6 in BPs), or during their transition to another progenitor type (e.g., Tbr2 in APs). Abbreviations: NE=neuroepithelial cells, RG=radial glia, BP=basal progenitors, bRG=basal radial glia, SNP=short neural precursors, TAP=transit amplifying progenitors.

|                        | NE            | RG                  | BP        | bRG       | SNP  | TAP   |
|------------------------|---------------|---------------------|-----------|-----------|------|-------|
| Apical contact         | ✓             | ✓                   | ✗         | ✗         | ✓    | ✗     |
| Basal contact          | ✓             | ✓                   | ✗         | ✓         | ✗    | ✗     |
| Transcription factor   | Pax6/<br>Sox2 | Pax6/<br>Sox2/ Tbr2 | Tbr2/Pax6 | Pax6/Sox2 | Pax6 | Tbr2  |
| Self-renewing capacity | high          | high                | low       | high      | ?    | high? |

cortical wall. As the apical surface of the neuroepithelium lines the ventricle of the developing brain, the zone is thus termed the **ventricular zone** (VZ) (Boulder Committee et al., 1970). With the onset of neurogenesis and the diversification of neural progenitors into distinct populations, the cortical wall separates into discernible germinative zones (Figure 1A).

RG cells, due to their highly elongated, somewhat “division-unfriendly” shape, require a highly regulated mitosis and cytokinesis. These control processes have to be tightly regulated in order to divide the cell properly and either expand the population of RG cells, or make neurons and other neural progenitor populations.

### **Basal/intermediate Progenitors**

At the onset of neurogenesis in mice (around E10 in the mouse), APs start producing another type of neural progenitor destined to leave the VZ (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Once an AP is committed to a lineage other than the proliferative one (either it will produce neurons or other types of progenitors) it starts expressing the antiproliferative gene *Tis21* (Haubensak et al., 2004; Iacopetti et al., 1999). These new neural progenitors lose contact with the ventricle and delaminate from the apical adherens junction belt, at the same time retracting their apical and basal processes (Attardo et al., 2008). The loss of apical and basal contacts renders this cell population non-polar, which has an impact on many of their properties (Fietz and Huttner, 2011). As mentioned, the newborn daughter cells migrate away from the apical surface, basally, and are referred to as either **basal progenitors** (BPs) (Haubensak et al., 2004) or **intermediate progenitors** (Noctor et al., 2004) (Figure 1C). The clustering of BPs in the zone adjacent to the ventricular zone makes up a new germinative zone called the **subventricular zone** (SVZ) (Boulder Committee et al., 1970).

BPs radically change their expression profiles (Arai et al., 2011; Kawaguchi et al., 2008) compared to APs. They lose the characteristics of RG and start expressing a set of proteins specific for this population. A marker protein that is expressed in BPs is *Tbr2* (Englund et al., 2005). BPs, as opposed to APs, do not possess a high self-renewing potential (possibly related to their lack of apical-basal polarity), so the presence of *Tbr2* marks the cell as committed to a neuronal lineage. BPs undergo mitoses in the SVZ and in 90% of cases, indicated by *Tis21* expression (Attardo et al., 2008; Farkas et al., 2008; Haubensak et al., 2004), produce two neurons in a self-consuming manner (Table 1).

## Basal Radial Glia

APs can also give rise to yet another type of neural progenitor. This population has only recently been described in greater detail (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011), but its existence has been noted for some time (Smart et al., 2002). After the RG division on the apical surface, the daughter cell delaminates from the ventricle in a similar manner as BPs. The crucial difference in this cell population is that the basal contact is retained. In addition to the retention of the basal contact and hence polarity, these cells continue to express the markers of RG (namely Pax6 and cytoplasmic glial markers) (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011; Shitamukai et al., 2011; Wang et al., 2011) (Table 1). Given that they still resemble radial glial cells, but are found at a more basal position than RG cells (in the SVZ) they have been named **basal radial glia** (bRG) (Figure 1C) (Kelava et al., 2012).

Due to the loss of apical contact, bRG migrate basally and populate the SVZ. In some species the rate of production of these cells is so high that, upon migration to the SVZ, they subdivide the SVZ into an **inner-SVZ** (ISVZ) and **outer-SVZ** (OSVZ), which are both made out of a mixture of BPs and bRG, but are distinguished by the density and orientation of nuclei (Smart et al., 2002). The relative abundance of the latter two neural progenitor types and the presence or absence of the subdivision of the SVZ is thought to be related to the vastly different neuronal output in different mammalian species (Fish et al., 2008; Kriegstein et al., 2006; Lui et al., 2011; Molnar et al., 2006; Smart et al., 2002).

## Short Neural Precursors and Other Neural Progenitors

The aforementioned neural progenitor types have been studied in great detail. Nonetheless, we do not presume that the developing neocortex is comprised only from these cell types.

An interesting additional neural progenitor population are the **short neural precursors** (SNPs) (Gal et al., 2006; Stancik et al., 2010) (Figure 1C). SNPs are, as their name implies, non-elongated progenitor cells residing in the VZ, with their basal process not reaching the basal surface, but ending somewhere in the VZ/SVZ. Little is known about the cell biological characteristics and self-renewing potential of these cells, but it is presumed that many of them divide symmetrically to produce two neurons (Gal et al., 2006). The only molecular marker distinguishing them from other APs found to this day is the active tubulin  $\alpha 1$  promoter (Gal et al., 2006).

Also in the case of the SVZ, it is highly possible that additional neural progenitor populations exist. One, the existence of which has been proven by live imaging but the cell biological features are still unknown, is the

**transient amplifying progenitor** (TAP) cell population (Figure 1C). These cells are presumably born from bRG in the SVZ and are able to self-renew (Hansen et al., 2010), but how they are related to the BPs characterized in mouse and rat is yet to be determined.

This chapter does not cover any form of gliogenesis, but it is assumed that special populations of gliogenic progenitors reside mostly in the SVZ and contribute to the heterogeneity and complexity of the germinative zones.

## **FATE DETERMINATION VIA THE MODE OF CELL DIVISION**

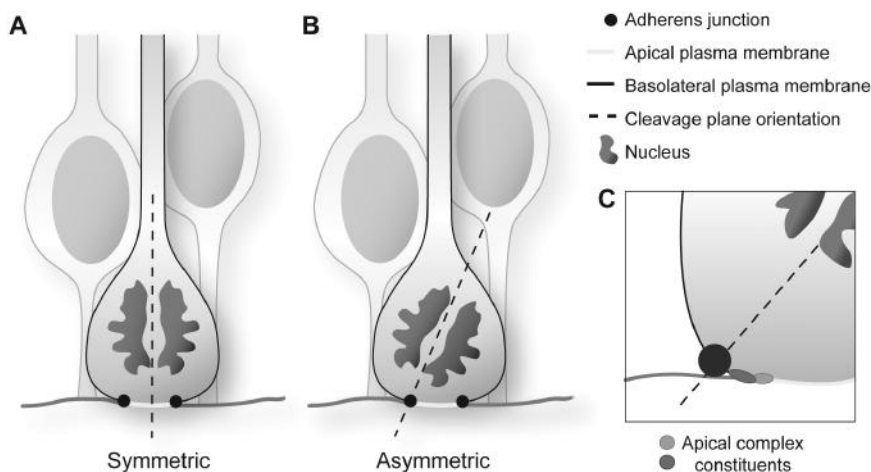
The development of the brain relies heavily on the tight control of the division of neural progenitors. With such an elongated cell shape, minute differences in mitotic spindle orientation change the cellular components inherited by daughter cells and thus influence cell fate.

Multiple studies have shown that, as neurogenesis progresses, the neural progenitors switch from a symmetric, proliferative mode of division to an asymmetric, neurogenic mode (Chenn and McConnell, 1995; Haubensak et al., 2004; Konno et al., 2008; Kosodo et al., 2004; Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2004; Shitamukai et al., 2011). As briefly mentioned in the overview of neural progenitor populations, there seems to be a strong connection between the presence of cell polarity and the self-renewing potential of different progenitor populations. Here we summarize the characteristics of these two principal types of divisions and give possible outcomes of either mode of division on a given cell type.

### **Symmetric Cell Divisions**

When mentioning symmetric cell division in the context of neural progenitors in the developing brain, it is very important to understand the relationship between the orientation of the mitotic spindle and apical-basal polarity of the progenitor cell. As in other systems, a symmetric division implies that both daughter cells receive similar amounts of the same cell fate-determining constituents (Figure 2A).

Before the onset of neurogenesis, NE cells divide rapidly and symmetrically in order to exponentially increase the number of progenitor cells that can later give rise to other neural progenitor types and, subsequently, neurons. Sometimes, during these early divisions, the basal process can be split in two, starting from the basal side, and moving apically, together with the cleavage furrow (Kosodo et al., 2008).



**Figure 2. Symmetric *versus* asymmetric divisions of RG.** (A) Magnification of the apical portion of the neuroepithelium with a RG cell dividing symmetrically. Note the symmetric inheritance of the apical membrane. (B) A RG cell dividing asymmetrically. Note the asymmetric inheritance of the apical plasma membrane and a portion of the adherens junctions complex by the daughter cells. (C) A magnification of the RG cell dividing asymmetrically. Some parts of the apical complex are not inherited by one of the daughters. This asymmetry may lead to a difference in fate between daughter cells.

BPs also exhibit symmetric cell divisions, as they do not possess apical-basal polarity. Almost all divisions of basal/intermediate progenitors yield two neurons, which migrate basally using basal processes of RG cells as scaffolds and settle in the growing cortical plate (Rakic, 1978).

The bRG-derived TAPs are capable of undergoing symmetrical division, giving rise to two TAPs, or eventually to two intermediate progenitors which in turn give rise to two neurons each (Hansen et al., 2010).

## Asymmetric Cell Divisions

Concomitant with the transition of NE to RG cells, asymmetrical divisions begin and this coincides with the generation of the first neurons in the cortex (Fishell and Kriegstein, 2003). Early work on divisions in the developing brain (Chenn and McConnell, 1995) correlated the appearance of horizontal cleavage planes (i.e., divisions in which the mitotic spindle is parallel to the apical-basal axis) with neuronal production. Later work (Konno et al., 2008; Kosodo et al., 2004), however, showed that, due to the peculiar shape of RG cells, even vertical divisions (i.e., divisions in which the mitotic spindle is perpendicular to the apical-basal axis), previously thought to

be symmetrical, actually represent asymmetrical divisions and give rise to different daughter cells, due to uneven inheritance of the polarized, apical-basal, cell constituents (Figure 2B).

The apical domain of the RG cell comprises a very minute fraction of the total plasma membrane (Kosodo et al., 2004), and the specific protein and lipid constituents that are thought to have an influence on the daughter cell fate are concentrated in this restricted area. Tilting of the cleavage plane just a few degrees away from 90° already bypasses the apical membrane and adherens junctions and causes the asymmetrical inheritance of these apical constituents and a difference in daughter cell fate (Figure 2C). The daughter cell which inherits the apical compartment has been thought to remain a RG cell and retain its self-renewing ability, while the daughter cell which does not, has been thought to undergo a change of fate and either become a neuron, or another type of neural progenitor (Kosodo et al., 2004). However, subsequent work in zebrafish has suggested the opposite relationship between apical domain inheritance and daughter cell fate (Alexandre et al., 2010; Dong et al., 2012).

In addition to apical constituents, time-lapse imaging studies (Konno et al., 2008; Shitamukai et al., 2011) implicated the basal compartment as being important for retaining self-renewing ability. Their experiments showed that, for APs to retain their self-renewing potential, it is necessary to inherit *both* the apical and basal compartment. The exact requirements that a daughter cell needs in order to sustain proliferative capacity are not yet fully understood, but recent work shed more light on the outcomes of different divisions in the VZ. The vertical divisions produce a RG and a neuron, while oblique divisions give rise to a RG and a BP, which continues producing neurons through indirect neurogenesis (Postiglione et al., 2011). A cytoplasmic protein Inscuteable (Insc) is involved in this regulation of cleavage plane (Knoblich, 2008; Postiglione et al., 2011).

Although they are polar (actually, monopolar, as they lose the apical but retain the basal contact), bRG cells do not show a tight control of division orientation. They divide mostly asymmetrically, producing one daughter bRG cells and either one neuron or a TAP (Fietz et al., 2010; Hansen et al., 2010).

There are still many unresolved issues as to the molecular mechanisms which control the switch from symmetrical proliferative to asymmetrical differentiative/neurogenic division. It is likely that this switch will result from a complex interplay between intrinsic factors and extrinsic cues.

## **APICAL SUBCELLULAR STRUCTURES IN NEURAL PROGENITORS**

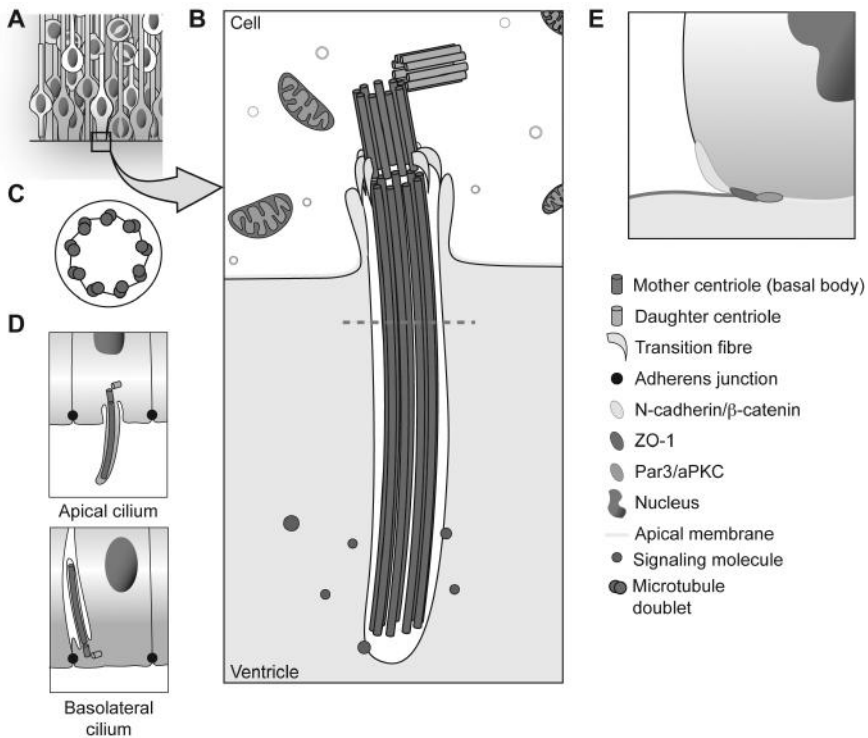
We have repeatedly mentioned the importance of cell polarity for the self-renewing potential of neural progenitors. In this section, we will focus on the apical compartment of neural progenitors and dissect the subcellular structures that might play a role in determining the mode and outcome of their divisions.

### **Primary Cilium**

As all epithelial cells exhibiting apical-basal polarity, APs have a primary cilium protruding from their apical surface (Figure 3A). The primary cilium of APs is a non-motile structure (motile primary cilia in the brain are present only in ependymal cells in the adult brain and in some choroid plexus cells) projecting into the ventricular space. These primary cilia have a 9+0 microtubule structure (Goetz and Anderson, 2010; Han and Alvarez-Buylla, 2010) (Figure 3B). The positioning of the cilia at the apical surface requires their anchoring structure, the basal body, to be at the same place. As the basal body represents the mother centriole of the centrosome, the apical location of the primary cilium has important implications for a process typical of APs—interkinetic nuclear migration.

The primary cilium is a structure underlying active signal transduction, and is thought to play a very important role in receiving signals from cerebrospinal fluid (CSF) and regulating the proliferative state of neural progenitors which contact the ventricle. The appendages which dock the basal body to the membrane represent a diffusion barrier and make the primary cilium a separate compartment in the cell. Therefore, any signals received by the cilium will be processed at the tip and transduced through the base into the cytoplasm (Louvi and Grove, 2011). The primary cilium is an essential part of Hedgehog (Hh) and Wnt signaling pathways in neural progenitors.

Although it was thought that the primary cilium of APs is always situated at the apical surface, a recent study showed that it can also be present at the basolateral membrane of neural progenitors exhibiting apical-basal polarity (Wilsch-Bräuninger et al., 2012) (Figure 3C). The occurrence of basolateral cilia correlates with the rate of neurogenesis. It is thought that these basolateral cilia are present on soon-to-be BPs, which did not yet delaminate from the ventricular surface. The process might be related to the differential inheritance of mother and daughter centriole-containing centrosomes.



**Figure 3. Primary cilium and the apical complex of RG.** (A) A portion of the cortical wall shown in greater detail in other panels. (B) The primary cilium (green), growing out of the basal body (the mother centriole) protrudes into the ventricle. (C) A section through the cilium (dashed line) reveals the arrangement of microtubule doublets. (D) Positioning of the apical cilium in an AP (top). Basolateral cilium in a soon-to-delaminate AP (bottom) (Wilsch-Bräuninger et al., 2012). (E) Schematic of the disposition of apical complex constituent in the apical portion of the AP.

*Color image of this figure appears in the color plate section at the end of the book.*

Although the contribution of cilium and/or ciliary based signaling to cell fate determination of neural progenitors is not known in great detail, the importance of this structure is reflected in diseases which are caused by mutations of ciliary or cilium-connected proteins. These disorders very often include malformations of the cerebral cortex and/or cognitive disorders (Gerdes et al., 2009; Han and Alvarez-Buylla, 2010; Louvi and Grove, 2011; Novarino et al., 2011).



## Centrosomes

Centrosomes are cytoplasmic structures crucial for microtubule organization and cell division. In the context of APs, centrosome positioning plays a pivotal role in cell fate determination, as it depends on the positioning of the mitotic spindle whether their division will be symmetric or asymmetric.

During AP interphase, the centrosome's older centriole, the so-called mother centriole, is docked to the apical plasma membrane by appendage proteins, constituting the basal body of the primary cilium. During S-phase of the cell cycle, the centrosome's two centrioles duplicate to generate, together with the pericentriolar matrix, two centrosomes, both of which are still tethered to the apical plasma membrane. As APs enter mitosis, the primary cilium is dismantled and the two centrosomes migrate to opposite poles of the dividing cell, typically the lateral plasma membrane, from where they will establish the mitotic spindle (Bettencourt-Dias et al., 2011; Siller and Doe, 2009). Recently, novel evidence concerning the differential inheritance of the mother *versus* daughter centriole-containing centrosomes in the context of daughter cell fate has emerged (Wang et al., 2009). It has been shown that, after asymmetric division of an AP, the daughter cell that inherits the centrosome with the mother centriole will remain an AP and retain the self-renewing capacity, while the other daughter cell will switch to a neuronal lineage, becoming either a neuron straight away (direct neurogenesis), or transforming into a BP (indirect neurogenesis). The molecular mechanisms driving this process remain to be elucidated.

Numerous proteins have been implicated in the positioning of the mitotic spindle during cell division. Of special interest for neural progenitors are the so-called **microcephaly** genes. Mutations in these genes cause a disorder called primary microcephaly (Bond et al., 2002; Buchman et al., 2010; Jackson et al., 1998; Nicholas et al., 2010). Microcephaly patients have a reduced brain volume with some form of mental retardation (Woods, 2004). An interesting feature of some of these genes is that they show signs of positive selection, which implies that during the course of evolution, a certain protein sequence is established in the population much faster than it would be by genetic drift, because the phenotype that this sequence produces is selectively beneficial for the organism (Evans et al., 2005; Mekel-Bobrov et al., 2007; Ponting and Jackson, 2005). It is therefore thought that these proteins, with their engagement in the decision between proliferative versus neurogenic divisions of neural progenitors, notably APs, might have played an important role in the expansion of the brain during the course of mammalian evolution (Fish et al., 2008; Fish et al., 2006).

## **Apical Junctional Complex and Cell Cortex**

The last structure in the apical compartment of neural progenitors that we will discuss is the junctional complex and associated cell cortex (in short referred to as apical complex). Before the onset of neurogenesis, NE cells possess tight as well as adherens junctions, but after the onset of neuronal production, the former are downregulated, and only adherens junctions remain (Götz and Huttner, 2005). Interestingly, a typical component of tight junctions, ZO-1, remains as a part of the apical adherens junction belt (Aaku-Saraste et al., 1996).

The apical complex contains, besides ZO-1, Par3, Par6 and aPKC, which are situated at the apical-most part of the cell, and  $\beta$ -catenin and N-cadherin, which are located more basally at the transition from the apical to the basolateral membrane, where they form an adherens junction belt (Figure 3D). This belt is a key feature of the apical-basal polarity of APs. The location of the constituents of the apical complex makes them potentially also differentially inherited during AP division. Indeed, it has been shown that Par3 is asymmetrically inherited during asymmetric divisions of APs (Kosodo et al., 2004) (Figure 2C). Further studies on the apical complex have shown that the amount of apical complex proteins has an effect on the cell fate (Costa et al., 2008), and that this affects Notch signaling (Bultje et al., 2009). As interference with N-cadherin has no effect on the rate of neurogenesis (Zhang et al., 2010), it is thought that it is just the apical proteins that are inherited asymmetrically and contribute to cell fate decision, while the adherens junction constituents are distributed equally and maintain the positioning of the cells after cytokinesis (Marthiens and French-Constant, 2009).

## **INTRINSIC CUES FOR FATE DETERMINATION**

What is it that drives a neural progenitor to continue proliferating or change fate into a neuron or a distinct, lineage-wise downstream, type of neural progenitor? The cues can come from the environment or from the neural progenitor itself. In the following section, we will describe the intrinsic molecular determinants of neural progenitors which have an impact on cell fate choice.

### **Transcription Factors**

Transcription factors are proteins which bind to specific genomic DNA sequences and can either promote or block the transcription of target genes. They play an indispensable role in the regulation of the state of the cell.

Many transcription factors have been found to be present in neocortical progenitors, so we will list only the most important ones, which are also used as molecular markers for different progenitor populations.

One of the first transcription factors appearing in the developing neuroepithelium is Pax6 (Paired-box 6). Pax6 is detectable in the neuroepithelium as early as E8.5 in the mouse (Walther and Gruss, 1991). This highly conserved transcription factor is involved in the development of the central nervous system in both embryonic and adult brain (Osumi et al., 2008), and its loss causes severe deformations in the brain, misspecifications of cortical neurons, complete loss of eyes and death soon after birth (Schmahl et al., 1993; Stoykova et al., 1997; Stoykova et al., 2000; Tarabykin et al., 2001). Pax6 is expressed in APs (Götz et al., 1998) and bRG (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011) (Table 1) and appears to perform a complex, dosage-dependent function. The dissection of Pax6 functions showed that it represses genes of the lateral ganglionic eminence in the neocortex and prevents the premature differentiation of progenitor cells (Quinn et al., 2007). In addition to promoting proliferation (Estivill-Torrus et al., 2002; Georgala et al., 2011) it appears that Pax6 also regulates neurogenesis and production of BPs (Quinn et al., 2007; Sansom et al., 2009). The complex role that Pax6 plays in neocortical development seems to maintain the optimal balance between proliferation and neurogenesis (Sansom et al., 2009). The exact mechanisms are still not yet fully elucidated, but an attractive insight into Pax6 function came from analyzing the cell divisions in Pax6 mutants. In these mutants, divisions of APs are altered, as compared to wild type, and this leads to a reduced pool of progenitor cells (Asami et al., 2011). So, in addition to transcriptional regulation of proliferative and neurogenic genes (e.g., *Hes1*, *Neurog2*, *Mash1*), Pax6 influences the genes which are connected to the orientation of the mitotic spindle, thus also contributing to the balance between symmetric and asymmetric divisions and, consequently, to the ratio of proliferative to neurogenic divisions.

As mentioned, one of the roles of Pax6 is to regulate the transition of APs to BPs. The most widely-used molecular marker for this progenitor population is *Tbr2* (T-brain 2). *Tbr2* is expressed specifically by BPs and its expression starts already in the VZ, where BPs are born and from which they will migrate to the SVZ (Englund et al., 2005) (Table 1). The fact that *Tbr2* function is limited to BPs is supported by studies showing that *Tbr2* conditional knock-out mice show a reduced abundance of BPs (decrease in mitoses in the SVZ), without an overt influence on APs (Arnold et al., 2008; Sessa et al., 2008). In addition, *Tbr2* is not expressed by bRG, which—in contrast to most BPs in mouse—exhibit self-renewing potential, but is found in human TAPs (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011). *Tbr2*-positive BPs produce most neurons in the developing mouse

neocortex by symmetric divisions (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004), and also contribute to the colonization of the neocortex by interneurons, which are born in the ventral telencephalon, by secreting chemoattractants (Sessa et al., 2010).

After the division of BPs, newly-born neurons start expressing another T-brain protein—Tbr1. Tbr1 is involved in the differentiation of the early-born neurons destined for the deep neuronal layers, which in turn have an impact on the correct specification and positioning of the later-born neurons destined for the upper neuronal layers (Hevner et al., 2001). The sequence of expression Pax6 → Tbr2 → Tbr1 characterizes the lineage RG → BP → neuron (Englund et al., 2005) and the different proliferative potential of these distinct cell populations.

Many other transcription factors are involved in the regulation of neurogenesis. We will just briefly mention some of them. Insm1 (Insulinoma-associated 1) is a zinc-finger transcription factors which is a key component of the machinery driving AP → BP transformation and is a positive regulator of Tbr2 (Farkas and Huttner, 2008). AP2γ is expressed in APs, is regulated by Pax6 and also controls the formation of BPs (Pinto et al., 2009). Sox2 (Sry-box 2), a transcription factor essential for maintenance of the proliferative state of embryonic stem cells, is expressed in APs and bRG, and seems to influence the proliferative state of these cell, but the underlying mechanism and its interaction with the other master regulator, Pax6, is still unclear (Bani-Yaghoub et al., 2006; Graham et al., 2003; Hansen et al., 2010; Kelava et al., 2012).

## **Regulatory RNAs**

The study of regulatory RNAs as an independent field of investigation was influenced by two major findings: (i) only 1–2% of the human genome encodes proteins; and (ii) many portions of the genome are transcribed, but not translated (Mattick, 2001). The advances in this novel field have uncovered an essential role for regulatory RNAs in the developing brain.

There are several classes of regulatory RNA, but the ones most studied in the context of brain development are micro-RNAs (miRNAs). These RNAs are 18–30 nucleotides long and bind to complementary sequences on target mRNAs (Li and Jin, 2010). Several miRNAs show brain-specific expression patterns: miR-9, miR-124a, miR-124b and miR-135. Studies on specific miRNAs showed that they have a role in regulating the expression of proneural genes in neural progenitors (Fineberg et al., 2009). Overexpression of miR-9 and miR-124 lead to the premature differentiation of progenitor cells (Krichevsky et al., 2006; Lim et al., 2005; Makeyev et al., 2007; Shibata et al., 2008). Different miRNAs have different pathways of suppressing non-

neuronal genes. miR-9, for example, regulates the expression of forkhead protein G1 (Shibata et al., 2008).

Studies on the protein machinery that produces functional miRNAs showed that, although important, miRNAs are dispensable for the expansion of the neural progenitors. Conditional knock-out of Dicer (an RNase III enzyme that cleaves miRNA precursors to produce mature miRNAs) showed no influence on the divisions of neural progenitors, but did have an impact on the survival and migration of newly-born neurons (De Pietri Tonelli et al., 2008). These studies show that a major role of miRNAs in the development of the brain is the “fine-tuning” of different pathways.

Along with miRNAs, the non-translated transcriptome contains transcripts which are much longer than 30 nucleotides. These RNAs have been referred to as long non-coding RNAs (lncRNAs). lncRNAs are usually more than 200 nucleotides long (Ponting et al., 2009) and are also presumed to play an important role in the regulation of development, with specific functions in the development of the brain (Mercer et al., 2008). lncRNAs exhibit functions which are similar to classically defined enhancers (Ørom et al., 2010) and are implicated in the regulation of stem cell self-renewal potential (Guttman et al., 2011). Whether lncRNAs perform a single major role in brain development, or are functionally diverse like protein-coding genes, is still not established. However, because it is known that lncRNAs may influence the expression of protein-coding genes (Huarte and Rinn, 2010) and that some lncRNAs show sequence and spatial expression conservation across vertebrates (Chodroff et al., 2010; Ponjavic et al., 2009), it is apparent that they are an important element in the normal development of the brain. In addition to their contribution to the development of the brain, lncRNAs might have also been instrumental to the evolution and expansion of the neocortex. It has been shown that human lncRNAs implicated in neural development show signs of accelerated evolution (Pollard et al., 2006). This gives us some indication of the mechanisms leading to the vast enlargement of the brain in the human lineage.

## **Signaling Pathways**

Probably the best-known signaling pathway which plays a role in the maintenance of stem cells is Notch. The canonical Notch signaling involves the differential expression of Notch ligands in a population of cells. Cells which express higher levels of ligands (Delta/Dll, Jagged) induce a stronger response of Notch in neighbouring cells and initiate the expression of downstream genes (Hes1, Hes5). Hes1 and Hes5 repress the expression of Notch ligands by transcriptional feedback, thereby blocking the possibility of the cell to induce Notch signaling in its neighbouring cells. Hes1 and Hes5 also repress the expression of proneural genes and through this effect keep

the cell in a stem cell state (Lui et al., 2011; Pierfelice et al., 2011). In this way, a heterogeneous population of cells in a given tissue is formed.

The importance of Notch signaling in brain development has been shown by various approaches. The overexpression of NICD (intracellular domain of Notch—the part of Notch protein that is cleaved off upon contact with the ligand, and which is transported to the nucleus, where it influences expression of downstream genes) resulted in increased number of cells expressing RG markers (Gaiano et al., 2000). Conversely, the disturbance of the Notch ligand processing enzyme Mindbomb 1 (MIB1) by a conditional knock-out showed a depletion of RG cells and an increase in neuronal production prematurely (Yoon, 2008). As MIB1 is expressed in BPs, rather than RG, this is a good example of the influence of other progenitor types on the proliferative state of APs (see below).

The level of Notch in RG cells oscillates with the phases of the cell cycle, as measured by the expression level of its downstream gene *Hes1* (Shimojo et al., 2008). If mitosis occurs during period of low *Hes1* in the cell, neuronally-committed progeny will be produced. The role of these oscillations is to maintain the pool of self-renewing progenitors, whilst at the same time producing neurons. Alongside the role as a mechanism for maintaining proliferative capacity in the VZ, Notch signaling also influences bRG cells. This is reasonable, as bRG cells, after being born from APs, retain the ability to self renew, as opposed to most BPs. Interference with the enzyme that cleaves off the NICD of Notch ( $\gamma$ -secretase) by the chemical inhibitor DAPT in the human OSVZ resulted with a decrease in the expression of proliferative genes, and an increase in neurogenesis (Hansen et al., 2010). In addition to that, it has been shown that BLBP (a RG marker expressed by APs and bRG cells) is a direct target of Notch signaling, thus confirming the involvement of Notch in the maintenance of the proliferative state of neural progenitors (Anthony et al., 2005).

The canonical Notch pathway seems to be present in only a subset of progenitor cells in the VZ—the APs (Mizutani et al., 2007; Oishi et al., 2004), and in bRG cells (Hansen et al., 2010). The other progenitor populations appear to transduce Notch signaling in a different way (Mizutani et al., 2007).

A plethora of different signaling pathways, in addition to Notch, operate in the expanding neuroepithelium. Signaling which utilizes secreted glycoproteins of the Wnt family is another important pathway in neural progenitors. Wnt signaling can take place through different routes. The canonical,  $\beta$ -catenin-mediated signaling pathway seems to be the predominant form of Wnt signaling in the developing brain. As opposed to Notch signaling, the role of Wnt signaling in the developing neuroepithelium is less clear. Several studies with contrasting results led to opposing views on the function of Wnt signaling in neural progenitors. Some studies (Chenn

and Walsh, 2002, 2003; Woodhead et al., 2006; Wrobel et al., 2007; Zechner et al., 2003) reported that the Wnt- $\beta$ -catenin pathway promotes proliferation of neural progenitors during early and mid-neurogenesis. One study specifically showed a peculiar phenotype. The expression of a dominant-active  $\beta$ -catenin, which lacked the key phosphorylation sites needed for its destruction, making it independent of Wnt signals and constitutively active, led to an over-proliferation of neural progenitors in the VZ (Chenn and Walsh, 2002, 2003). Such an expansion of the neuroepithelium led to the folding of the apical surface and hypertrophy of the brain.

On the other hand, other studies (Hirabayashi and Gotoh, 2005; Hirabayashi et al., 2004; Israsena et al., 2004; Kuwahara et al., 2010) showed that Wnt signaling promotes differentiation of neural progenitors. An interesting discovery to this regard was that a transcription factor N-myc, which is involved in the control of proliferation and cancer, plays a role in both the Wnt-mediated proliferation and differentiation of neural progenitors (Kuwahara et al., 2010).

The aforementioned contrasting results might be due to the differential Wnt-signaling role in different populations of progenitors. It seems that the canonical Wnt/ $\beta$ -catenin pathway promotes proliferation in APs, while stimulating differentiation in the population of BPs (Munji et al., 2011). The exact basis of this differential function is yet to be unraveled.

We have previously highlighted a special compartment in the cell—the primary cilium and its role in signal transduction. An important segment of the signaling cascade taking place at the tip of the cilium is Hedgehog (Hh) signaling. The fact that Hh signaling has a fundamental role in the normal development and patterning of the brain is seen in Hh mutants which exhibit holoprosencephaly (a complete fusion of brain hemispheres) (Chiang et al., 1996; Rallu et al., 2002). Hh signaling takes place via its membrane receptor Patched (Ptc) and another membrane protein that is inhibited by Ptc in the absence of Hh, Smoothened (Smo), which upon Hh-binding-induced relief of inhibition by Ptc transduces Hh signals to the cytoplasm. Ptc and Smo have been detected in the developing embryonic neocortex (Zhu et al., 1999) but detection of Hh mRNA was problematic (Dahmane et al., 2001), which can be explained by the fact that most Hh signals come from the CSF.

Hh signaling in the embryonic neocortex regulates the proliferation of neural progenitors (Lien et al., 2006), probably by controlling cell cycle kinetics (Komada et al., 2008). Furthermore, conditional knock-out of Ptc, which leads to constant activation of Hh signaling, results in the expansion of neural progenitors and improper patterning (Dave et al., 2011). Conditional knock-out of Smo induces decreased proliferation, increased apoptosis and premature differentiation of neural progenitors (Komada et

al., 2008). Although Hh signaling was thought to be mostly connected to the apical side of the neuroepithelium, GABAergic neurons coming from the ventral telencephalon also influence the proliferation in the neocortex by producing Hh (Komada et al., 2008).

The need for precise regulation of proliferation *versus* differentiation in the developing neocortex requires orchestration of all signaling pathways in neural progenitor cells. That such intertwining of signaling pathways indeed occurs has been shown, for example, by the upregulation of Notch targets upon constant activation of Hh signaling (Dave et al., 2011) and by showing that the neurogenic effects of Wnt7a *in vitro* depend on Hh signaling (Viti et al., 2003).

Of course, additional signaling pathways are implicated in the development of the brain. We will just briefly mention a couple. Fibroblast growth factor (Fgf) signaling has been implicated in brain development on multiple levels. It has been shown that it has a role in pattern formation (Fukuchi-Shimogori and Grove, 2001; Gutin et al., 2006). It also influences progenitor proliferation (Raballo et al., 2000; Vaccarino et al., 1999; Vescovi et al., 1993) and cell-fate specification (Maric et al., 2007; Yoon et al., 2004). It was demonstrated that Fgf signaling pathway interacts with Notch signaling (Ever et al., 2008; Yoon et al., 2004). Another signaling cascade shown to function in brain development is ephrin signaling. Ephrin-mediated signaling has first been implicated in the control of axon guidance (Flanagan and Vanderhaeghen, 1998; Klein, 2004; Poliakov et al., 2004). Its involvement in brain development has recently been explained by its activation of proapoptotic pathways in early neural progenitors (Depaepe et al., 2005). This regulation of the number of progenitors results in a tight control of the final number of neurons and, therefore, of the size of neocortex. A recent addition to unraveling the function of ephrin signaling in brain development showed that it is necessary for the tangential migration of newly-born neurons and proper generation of cortical columns (Torii et al., 2009).

## EXTRACELLULAR SIGNALS

As any other tissue in a living organism, the developing brain is not an isolated system, and neural progenitors are constantly exposed to signals coming from the environment (ventricles, blood vessels, meninges). In the following section, we will describe the sources of signals, other than the intrinsic properties of the neural progenitors themselves, which can be found in the developing brain.



## Signals from the CSF

The cerebrospinal fluid (CSF) is a liquid that fills the spaces inside the brain—the ventricles, the spinal cord canal and subarachnoid space surrounding the central nervous system (Lehtinen and Walsh, 2011). But how would this fluid represent a source of signals which could influence the fate of cells it surrounds? Originally, CSF is in fact amniotic fluid that remains trapped in the cavities of the brain after closure of the neural tube. In addition to the constituents of amniotic fluid, CSF contains molecules secreted by the surrounding neuroepithelium and the choroid plexus (Dziegielewska et al., 1981; Parada et al., 2005; Zappaterra et al., 2007). The choroid plexus is a protrusion of modified ependyma into the ventricles, which appears with the onset of neurogenesis and is responsible for the secretion of most CSF components (Zheng and Chodowski, 2005). It is this rich and complex composition of the CSF that endows it with its signaling capacity.

From multiple studies, it has been established that the CSF contains a myriad of different proteins: proteins of the extracellular matrix, regulators of osmotic pressure, carrier proteins, hormone-binding proteins, regulators of lipid metabolism, enzymes and their regulators (Cavanagh et al., 1983; Parada et al., 2005), as well as Bmps (bone morphogenetic proteins), Wnts, Fgf8 (Fukuchi-Shimogori and Grove, 2003), Hh (in the cerebellum (Huang et al., 2009)), insulin and Igf1 and -2 (Lehtinen et al., 2011). CSF also contains membrane particles, notably prominin-carrying vesicles, which are thought to originate from the apical surface of the neuroepithelium (Marzesco et al., 2005). It has also been established that the CSF extracted from different parts of the brain (lateral ventricle and hindbrain ventricle) has a different composition of proteins (Zappaterra et al., 2007). Furthermore, choroid plexi from different parts of the brain have been shown to secrete distinct sets of proteins (Awatramani et al., 2003; Huang et al., 2009). In addition to these spatial differences, CSF changes composition through developmental time (Dziegielewska et al., 1981). It has been demonstrated that CSF extracted from the ventricles of the developing brain can support neural progenitors in neurosphere culture by itself, and the highest growth rate is accomplished when the cells are incubated with CSF of the same developmental stage (Lehtinen et al., 2011). This complex spatial and temporal organization of the CSF must have great impact on the neural progenitors which line the ventricle and influence their state, presumably through interaction of the various CSF constituents with receptors on the primary cilium and/or associated with the apical complex.

In addition to the signals it provides, other roles of CSF should not be neglected. As a continuous flow of fluid, which extends barrierless throughout the central nervous system, CSF is thought to have a synchronizing capacity (Lehtinen and Walsh, 2011). Also, CSF as a fluid

may exert pressure on the surrounding tissue (Desmond and Jacobson, 1977; Lowery and Sive, 2009; Pexieder and Jelinek, 1970), notably the cells lining the ventricles. It has been shown that increasing the pressure of the CSF leads to increased mitotic activity in the developing chick brain (Desmond et al., 2005). It is possible that a similar mechanism operates in the developing mammalian brain.

## **Signals from the Basal Lamina and Meninges**

In addition to the regulation taking place at the apical side of neural progenitors, there is also an elaborate relationship of neural progenitors to the tissues on their basal side. Again, this refers to cells which keep their basal contact, namely NE cells, RG cells and bRG cells.

The basal side of the cerebral cortex contacts non-neuroepithelial tissue—the meninges. Mesenchymal cells in the innermost meningeal layer, the pia, secrete constituents of the basal lamina (basement membrane), a “cell-free” layer of connective tissue, which covers the basal side of the neuroepithelium. NE cells also contribute to the formation of the basal lamina (Arai et al., 2011; Cohen and Hay, 1971; von Holst et al., 2007) by secreting its constituents. The membrane lying above the pia is the arachnoid. The arachnoid has an important role in regulating CSF on the basal side. The outer-most membrane is the dura, which is attached to the skull. These membranes, in addition to the protection and cushioning they provide for the brain, also have an impact on the cortical cells which lie below them, by regulating the attachment of their basal process and secreting different factors.

A very important factor secreted by the meninges is retinoic acid (RA). By using mutant mice that have a defect in forebrain meningeal formation (Foxc1 mutants), it has been shown that this RA has an important effect on the production of BPs and neurons (Siegenthaler et al., 2009). Meninges themselves can have an influence on the proliferation of neural progenitors. It has been demonstrated that meningeal cells stimulate neuroblast proliferation in cell culture (Barakat et al., 1981; Gensburger et al., 1986). In addition, the components of the basal lamina, notably integrins, also appear to affect neural progenitors and to be involved in the regulation of neurogenesis (Wojcik-Stanaszek et al., 2011). The role of neural progenitor—basal lamina attachment remains an open issue, since there are both studies showing that this attachment is necessary (Radakovits et al., 2009) or dispensable for the maintenance of neural progenitors (Haubst et al., 2006).

Besides basal attachment itself, the basal lamina also provides ligands for integrin signaling. There is a number of integrins expressed in the developing mammalian neocortex (Hirsch et al., 1994; Yoshida et al., 2003).

Specific blocking of integrin  $\alpha_v\beta_3$  by the viper venom echistatin reduced the number of cycling RG and bRG cells (meaning those progenitors with a basal contact) in ferret neocortex (see below) (Fietz et al., 2010). In addition, in the mouse, the RG that lose their apical contact do not exit the cell cycle but are able to continue cycling (Konno et al., 2008; Shitamukai et al., 2011). The ability of basal lamina-contacting cells to receive signals from another source except the apical side is thought to influence their self-renewing potential to a great extent and might have played a role in the expansion of the brain.

### **Signals from Blood Vessels**

The developing brain, as a particularly energy-demanding tissue, must ensure an appropriate supply of metabolites and oxygen. As the neuroepithelium is incapable of producing blood vessels, surrounding mesenchymal cells are recruited (Hogan et al., 2004). The pattern of blood vessel distribution becomes more complex as neurogenesis progresses and the cortical wall thickens, in order to match the metabolic demands of the growing tissue (Carney et al., 2007; Cheung et al., 2007). In the adult brain, a role for blood vessels in creating a distinct neurogenic niche has been established (Ohab et al., 2006; Palmer et al., 2000; Tavazoie et al., 2008).

In the embryonic brain, on the other hand, the work is still in progress. An interesting observation has been made concerning the distribution of mitotic cells in the SVZ. Mitotic, phosphohistone H3-positive cells, as well as Tbr2-positive BPs, are found closer to blood vessels than one would expect from a random distribution (Javaherian and Kriegstein, 2009; Stubbs et al., 2009). This finding speaks in favour of blood vessels creating a neurogenic niche for these neural progenitors, presumably by providing certain factors. Indeed, it has been shown that endothelial (but not vascular smooth muscle) cells release soluble factors that stimulate the proliferation of neural progenitors (Shen et al., 2004).

Two additional interesting facts reveal a tight link between neural progenitors and blood vessels: (i) many axonal guidance proteins also regulate developmental and neural angiogenesis (Carmeliet and Tessier-Lavigne, 2005), and (ii) telencephalic angiogenesis is under control of the same homeobox genes that regulate progenitor proliferation and differentiation (Dlx1/2, Nkx2.1, Pax6) (Vasudevan et al., 2008).

### **Signals from other Neural Progenitors and Neurons**

It is not only from non-neural tissue that neural progenitors can receive signals during their proliferative and/or differentiative phase. Other neural

progenitors and neurons also emit signals which influence the state of progenitor cells residing in the germinal zones.

The best understood interaction of neural progenitors with other progenitors is the Notch signaling. As Notch functions via direct cell-cell contact, it is logical that the surrounding cells will be the ones most involved in this type of signaling. Nevertheless, the peculiar shape of NE cells and the complex composition of the developing forebrain in terms of different progenitor populations, renders Notch signaling in the embryonic brain a bit less straightforward. Due to the highly elongated shape of RG cells, it is possible for them to receive signals from multiple germinal zones and cell types (other progenitors and neurons). Indeed, it has been shown that it is the population of BPs that are the major source of Notch ligands for RG cells (Campos et al., 2001; Yoon et al., 2008). One study (Yoon et al., 2008) connected a particular behavior of BPs called sojourning (the amount of time a BPs spends in the SVZ, before dividing and leaving the zone (Bayer et al., 1991; Noctor et al., 2004)); to their role in maintaining active Notch signaling in RG cells.

After a neuron is born, it does not stop interacting with its birthplace environment. While leaving the germinal zones, the newborn neuron continues to signal to the RG cell to which it is attached while migrating basally. There are indications that, once the young neurons are settled in their position in the cortical plate, they continue signaling to progenitor cells in the germinal zones through transmembrane proteins and/or secreted factors (Figiel and Engle, 2000; Ohta et al., 2006; Suh et al., 2001).

The neurons born in other parts of the brain (i.e., not in the neocortex) also contribute to making a neocortex-specific stem cell niche. During peak neurogenesis in the mouse, axons coming from the thalamus secrete a factor that promotes the proliferation of neural progenitors (Dehay et al., 2001). This factor shortens the cell cycle through reduction of the G1 phase, and facilitates G1/S transition. GABAergic neurons coming from the ventral telencephalon also influence proliferation of neocortical progenitors by releasing GABA (Wang and Kriegstein, 2009). A more striking neuronal fibre invasion of the developing neocortex is seen in primates (Smart et al., 2002). As previously mentioned, the SVZ of some animals can be subdivided into an ISVZ and an OSVZ. In primates, these two germinal layers are separated by an inner fibre layer. This fibre layer is composed of incoming axons and might play a role in maintaining the huge proliferative capacity of the primate SVZ. Apparently, in addition to supporting proliferation, secretions from neurons can also promote differentiation. Thus, a Bmp family member, produced by incoming axons, has been found to induce neurogenesis of neocortical progenitors (Chang et al., 2003).

## COMPLEX PROCESSES INVOLVED IN CELL FATE DETERMINATION

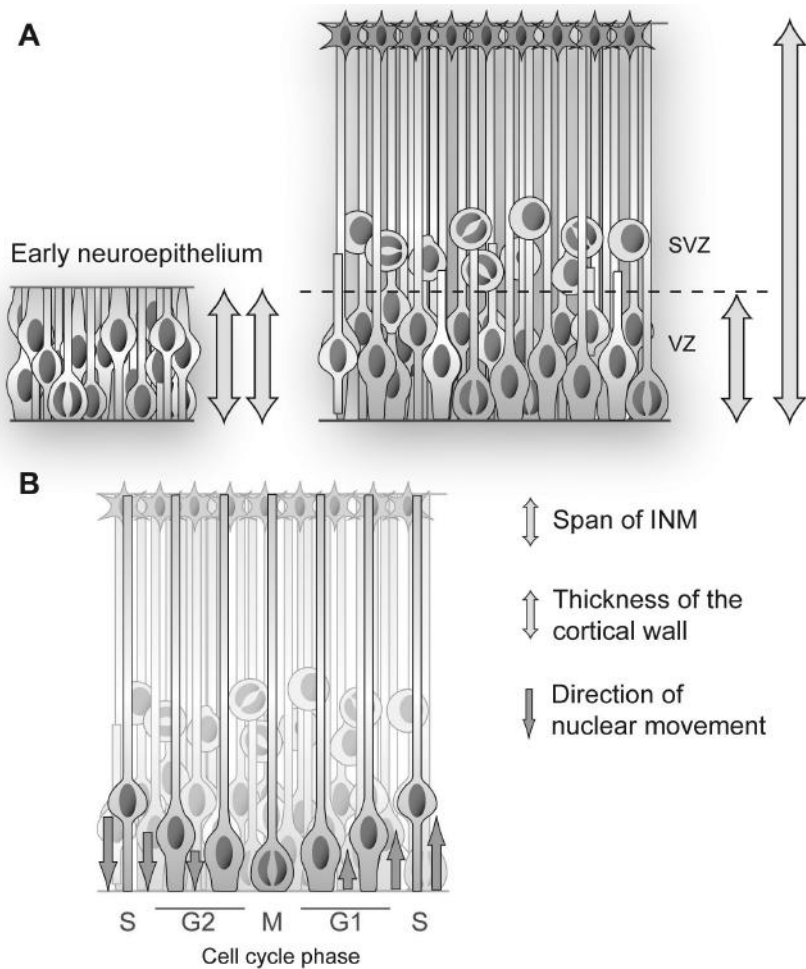
One would presume that extrinsic and intrinsic cues for cell fate determination suffice for the proper functioning of a progenitor cell and its balance between proliferation and neurogenesis. Yet, there are additional complex processes involved in the determination of cell fate. Here, we will discuss the two most prominent processes operating in the neuroepithelium: (i) interkinetic nuclear migration and (ii) cell cycle length.

### Interkinetic Nuclear Migration

Interkinetic nuclear migration (INM) is a hallmark of APs. Although previously thought to be characteristic of the mammalian neuroepithelium, studies on *Drosophila melanogaster* and the cnidarian *Nematostella vectensis* have shown that INM is likely a general feature of pseudostratified epithelia (Meyer et al., 2011) and the processes driving it are probably conserved across tissues and species.

INM is the organized movement of nuclei along the apical-basal axis in concert with the cell cycle (Taverna and Huttner, 2010). This orchestrated movement is what gives the neuroepithelium its characteristic pseudostratified appearance. The nuclei in G2 move towards the apical surface where they undergo mitosis. During G1, the nuclei move basally and during S phase the nuclei do not show much overt movement. In the developing neuroepithelium, we can distinguish two subtypes of INM: in NE cells (before the onset of neurogenesis), INM spans the whole thickness of neuroepithelium; after the transformation of NE cells into RG, and with the accompanying thickening of the cortical wall, the span of INM comprises only a portion of the RG apical-basal extension, with the basal-most limit of nuclear migration being the boundary of VZ to SVZ (Figure 4A). The principal reason behind both subtypes of INM is the previously described apical location of the centrosomes. In order to undergo division, APs must transfer their nuclei towards the apical domain of the cell (Figure 4B).

What are the molecular mechanisms driving this process? It has been shown that INM itself is not required for cell cycle progression, as interfering with INM does not disrupt entrance into mitosis (Baye and Link, 2008; Murciano et al., 2002). The same does not hold true for the opposite case. Studies on pharmacological treatments to arrest cells in G2/M phase have shown that this arrest inhibits INM (Baye and Link, 2008; Ueno et al., 2006). A growing body of evidence also shows that, mechanistically, the apical-to-basal and basal-to-apical movements of the nuclei are not the same processes.



**Figure 4. Interkinetic nuclear migration of APs.** (A) In the neuroepithelium at an early developmental stage INM spans the whole thickness of the cortical wall (left). After the onset of neurogenesis, when the cortical wall thickens and separates into germinal zones, the span of INM relative to the cortical wall thickness shortens (although absolutely it remains the same) (right). Colours and labels of progenitors as in Figure 1. (B) Direction of nuclear movement, depending on the phase of the cell cycle of an AP. Note that this scheme assumes that at least one of the daughter cells remains an AP which will continue undergoing INM.

*Color image of this figure appears in the color plate section at the end of the book.*

The microtubules in RG cells are oriented parallel to the apical-basal axis, with their plus ends directed away from the centrosome (Norden et al., 2009). Given their established role in organelle transport, microtubules have long been thought to provide a plausible mechanism for INM. Studies

conducted on neuroepithelia of different species have shown that the basal-to-apical movement of nuclei during INM (that is, before mitosis) involves the microtubule minus-end-directed dynein motor system (Del Bene et al., 2008; Gambello et al., 2003; Tsai et al., 2005; Tsuda et al., 2010). In this system, the nucleus is transported as cargo anchored to the microtubule motor system via the Syne2a protein (Zhang et al., 2009). A live-imaging based study on the zebrafish retina (Norden et al., 2009) also showed the contribution of actomyosin to the basal-to-apical movement.

When discussing apical-to-basal movement (after division), it is important to notice that this process, as well as serving in INM, is also involved in the delamination of newly-born BPs and bRG cells from the VZ, as well as the migration of neurons. Pharmacological interference using blebbistatin, an inhibitor of myosin II, showed that the apical-to-basal movement of progenitor nuclei involves actomyosin (Schenk et al., 2009). In this instance, the nucleus is not moved as a cargo but via directional myosin-dependent constriction. In addition to the actomyosin, it appears that microtubules also play a role in this movement. It has been suggested that plus-end-directed microtubule-based motors, but this time of the kinesin type, move the nuclei basally (Baye and Link, 2008; Tsai et al., 2010). The linkage is again provided by Syne2a, as it also has the ability to bind to kinesin (Zhang et al., 2009). In addition to these active movements, there is presumably a significant contribution of passive movements. One can imagine that the pressure exerted on the cell in such a densely-packed tissue like the neuroepithelium will have an impact on the positioning of the nucleus. In fact, a recent study (Kosodo et al., 2011) has shown that the nuclei which have just undergone mitosis at the apical surface are displaced basally by forces generated by other nuclei migrating towards the apical surface. The previously mentioned actomyosin-independent apical-to-basal movement could therefore be due to the indispensability of myosin for general cytokinesis (Kosodo et al., 2011).

What kind of effect could this nuclear migration have on the fate of the cell? There are a number of factors that show an apical-to-basal gradient. Positioning the cell body in different concentrations of a factor might have an impact on what kind of daughter the cell will produce after division as, for example, Notch is predominantly localized at the apical-most portion of the AP lateral plasma membrane. The interference with the descent of the nucleus (basal-to-apical movement), meaning that the AP nucleus spent more time in the basal portion of the VZ, where there is less Notch, resulted in premature AP differentiation (Del Bene et al., 2008). Thus, one function of INM would be to control the exposure of AP nuclei to signals originating in the apical domain of the cell.

Another possible function of INM could be related to space limitation. Due to the positioning of the centrosomes at the apical surface, AP nuclei

need to translocate to the apical surface to undergo division. As the ventricular surface is limited in space, it is necessary to maximize the space available for APs in mitosis, by moving the interphase nuclei out of the way. This spatial constraint of the apical surface might have also played a role in the evolution of other progenitor types, as the necessity to produce more neurons required more progenitor cells, but without vastly expanding the apical surface (Smart, 1972a, 1972b). An ingenious solution was to generate progenitor cells that divide away from the apical surface (although this loss of apical contact influences their proliferative capacity).

Recently, with the discovery of bRG cells, a peculiar phenomenon was described. Using live-imaging techniques, GFP-labeled bRG cell divisions in the SVZ of the developing human brain were monitored (Hansen et al., 2010). These cells exhibited a peculiar process termed “mitotic somal translocation”. During this, a bRG cell body moved basally before undergoing division, in a process which resembles INM. The significance of this curious process is still unknown.

## **Cell Cycle Length**

It has been shown that neural progenitors with different fate (proliferation *versus* neurogenesis) have distinct cell cycle length (Arai et al., 2011; Calegari et al., 2005; Caviness et al., 1995; Takahashi et al., 1995). Specifically, with the progression of neurogenesis, the cell cycle length of neural progenitors in the VZ increases. This reflects two distinct aspects of cell cycle regulation. First, APs at early stages of neurogenesis have a shorter cell cycle than APs at later stages (Caviness et al., 1995; Salomoni and Calegari, 2010). Second, at any given stage of neurogenesis, BPs have a longer cell cycle than APs, and while there are no BPs in the VZ prior to the onset of neurogenesis, they constitute ~30% of the neural progenitor nuclei in the VZ at mid-neurogenesis (Arai et al., 2011). Surprisingly, at one and the same stage of neurogenesis, APs committed to neurogenesis have a shorter cell cycle than those still proliferating. Lengthening of the AP cell cycle with progression of neurogenesis, and that of BPs relative to APs, reflects an increased length of G1 (Calegari et al., 2005; Salomoni and Calegari, 2010). This cell cycle phase is thought to be particularly important for the determination of daughter cell fate (Calegari et al., 2005; Dehay and Kennedy, 2007). A hypothesis as to how changes in cell cycle length might affect neural progenitor cell fate has been proposed (Calegari and Huttner, 2003). In support of this model, pharmacological lengthening of the AP cell cycle has been found to cause premature neurogenesis (Calegari and Huttner, 2003). Conversely, reducing G1 length by overexpression of cell cycle regulators in neural progenitors promotes their expansion (Lange et al., 2009; Pilaz et al., 2009).



In addition to the length of G1, it seems that changes in other cell cycle phases may also accompany changes in cell fate. Thus, S phase is longer in proliferating neural progenitors than those committed to the neuronal lineage (Arai et al., 2011). It is thought that this reflects the greater need of still-proliferating neural progenitors for DNA proofreading and repair after replication.

Moreover, the differences in cell cycle length are not just a feature of different populations of neural progenitors. Even different regions of the same brain can exhibit local differences in cell cycle length. This has been shown paradigmatically in the visual cortex of the macaque (Lukaszewicz et al., 2005). Two adjacent areas in the macaque cortex—A17 and A18—have distinct cell cycle parameters, with A17 progenitors having a shorter cell cycle and reduced G1 length. This is probably due to the specific expansion of A17 and the need for a larger number of neurons.

## **EVOLUTION OF THE NEOCORTEX**

The neocortex is a mammal-specific structure. It is thought to be homologous to the isocortex of birds and the dorsal cortex of reptiles (Aboitiz, 2011; Cheung et al., 2007), although these structures have a simpler, three-layered pattern. The appearance of a six-layered neocortex is thought to contribute to the higher cognitive functions that mammals possess (although several bird species are capable of highly complex behaviours and learning). In order to gain more insight into the process of brain evolution, it is necessary to undertake comparative neurogenesis studies (Abdel-Mannan et al., 2008; Cheung et al., 2010; Molnar et al., 2006).

## **Lessons from Atypical Model Organisms**

Most research conducted in the area of mammalian neurogenesis has been performed in rodents, mainly mice. Although mice have been an excellent model for studying neurogenic processes, one cannot deduce a “universal” principle of mammalian brain development from one species, as there exists no such thing as “universal mammalian model”. Indeed, an emerging body of evidence shows that the process of neurogenesis differs between species on both subtle and dramatic levels.

The first interspecies difference in mammalian neurogenesis was discovered by comparing the mouse and the macaque brain. In the developing macaque brain, there exists a large population of mitotic cells in a highly expanded SVZ (Smart et al., 2002). This fact, together with additional cytoarchitectonic differences and distinct pattern of incoming neuronal fibres, allowed the sub-division of the SVZ into the ISVZ and

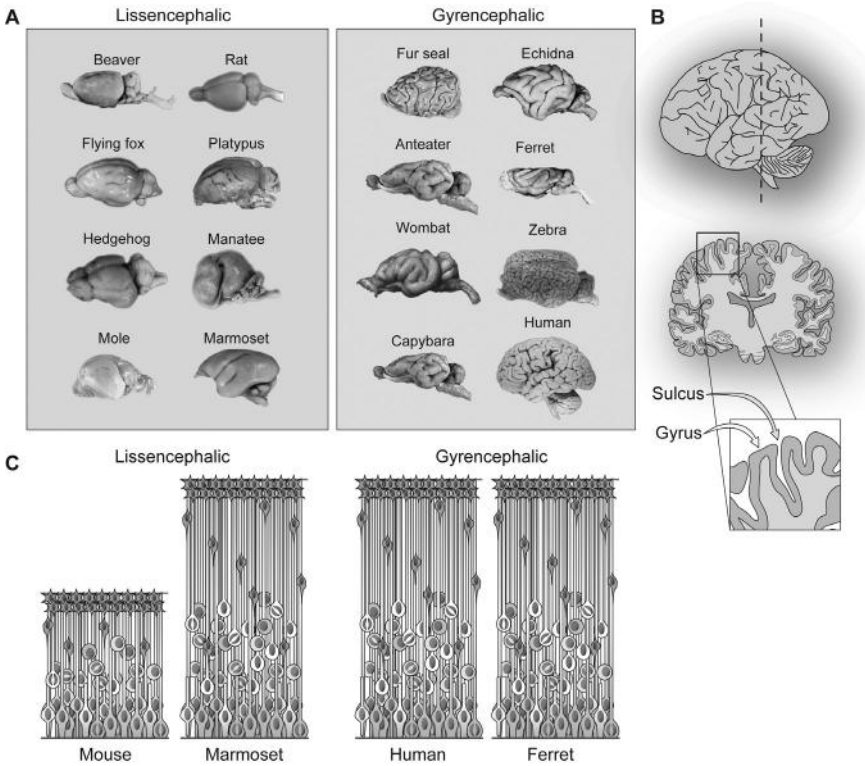
OSVZ. Further studies have shed more light on the cellular component of these novel zones and concluded that the OSVZ contains a novel progenitor type, the bRG (Fietz et al., 2010; Fish et al., 2008; Hansen et al., 2010). Although it was originally hypothesized that bRG cells were primate-specific, the discovery of bRG cells with a similar molecular profile and population density in the brain of a small carnivore, the ferret (Fietz et al., 2010; Reillo et al., 2011), challenged this view. As both the human and the ferret are gyrencephalic, it was postulated that bRG cells might be necessary for developing a gyrencephalic brain. This view, as discussed below, has also been questioned.

### **Lissencephalic vs. Gyrencephalic Brains**

Presented with various brains from different orders of mammals (Figure 5A), it is easy to notice the vastly different shapes and sizes. But on closer inspection, it is quite apparent that all of these brains can roughly be divided into two categories: the ones with a smooth surface—**lissencephalic**—and the ones with convolutions on the surface of the cortex—**gyrencephalic**. The difference between lissencephalic and gyrencephalic brains is that the gyrencephalic brains have a vastly expanded cortical surface (and thus a higher number of neurons) which must be compacted. The process of compacting a large surface into a cranium causes the brain to fold into numerous **gyri** and **sulci** (Figure 5B).

The notion that bRG cells can be found in the human, macaque and the ferret gave rise to the attractive idea that bRG cells are gyrencephaly-specific. Surprisingly, soon after this idea was published, two studies reported the existence of bRG cells in the developing mouse neocortex (Shitamukai et al., 2011; Wang et al., 2011). The mouse bRG cells appear identical to the ones found in the gyrencephalic species, yet there is a crucial difference. In the mouse, bRG cells comprise only 5% of progenitors in the SVZ, compared to 40–50% in gyrencephalic species (Figure 5C). These reports modified the hypothesis of bRG contribution to brain expansion. Now it is thought that it is not merely the existence of bRG cells, but also their relative proportion in the pool of progenitor cells, that is necessary for greater neuron production.

Yet another atypical model organism gave more insight into the complex nature of mammalian neurogenesis. The marmoset, a near-lissencephalic primate, possesses bRG cells at similar relative abundance in the SVZ as humans and ferrets (Garcia-Moreno et al., 2012; Kelava et al., 2012) (Figure 5C). The seemingly contradictory appearance of the high numbers of bRG cells and the lissencephalic appearance of the marmoset's neocortex could perhaps be explained by the process of dwarfing (Kelava et al., 2012). As the whole marmoset lineage decreased in size during evolution (Ford, 1980),



**Figure 5. Lissencephalic and gyrencephalic brains.** (A) A subset of brains belonging to species from different mammalian orders. Images were taken from the Comparative Mammalian Brain Collections ([www.brainmuseum.org](http://www.brainmuseum.org)). Note that images are not to scale. (B) Drawing of an adult human brain (top). The dashed line represents the area of a coronal section shown below. Note gyri and sulci of different sizes and depths. (C) Schematic of the cellular composition of the developing brains of a corresponding developmental age in several studied species (labels of progenitors as in Figure 1).

it has been postulated that the simplification of the gyrification pattern also followed as a consequence of a dwarfing brain. This again shows us that there are additional processes and features, other than number of progenitor cells, that can be changed in order to accompany the need of a species for a different brain. Likely candidates for these processes are: cell cycle length (it can be prolonged or shortened, in order to produce less or more neurons), the number of proliferative cycles a progenitor cell is able to undergo, the downstream fate of the progenitor cell (producing another type of cell that is able to self-renew, or exiting the cycle and become a neuron). Another process, which is easily overlooked, is the length of the neurogenic period. A longer neurogenic period will, as an outcome, produce more neurons. The later neurogenesis starts in the course of gestation, the

more precursor cells, and therefore neurons, will be generated (Charvet et al., 2011). The “tweaking” of these processes by heterochrony (change in the timing of an event) can lead to immense differences in the outcome of neurogenesis. Taken together, a developing mammalian brain can be seen as a set of modules, each of which can be changed in order to produce an optimal outcome.

In all supraorders of mammals we can find both lissencephalic and gyrencephalic species. This suggests that the precursors for gyrencephaly were already present in the last common ancestor of mammals, and that the brain is a very plastic organ, the anatomy and morphology of which can be changed with relative ease.

## **Models of Neurogenesis**

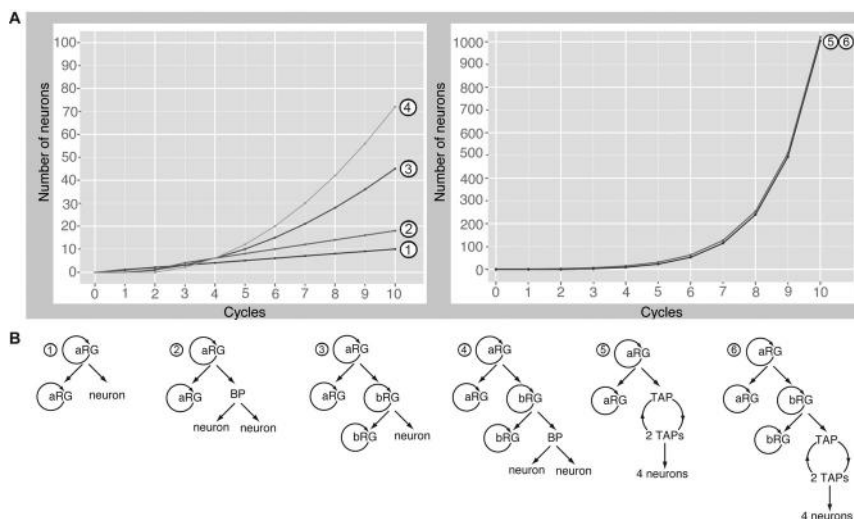
What are the advantages associated with modifying the types of progenitor cells in the brain or changing their numbers? If we examine the neurogenic *versus* proliferative potential of each progenitor type (Figure 6), we see that the addition of progenitor types with the ability to self-renew, along with producing neurons, has an enormous effect on neuron numbers.

The tight control of neuron production cannot be regarded as a “run-away” process in which it is the goal of the brain to make as many neurons as possible. As we have mentioned, sometimes a species has to decrease its neuron numbers in order to satisfy some other constraints imposed by the environment. Furthermore, the number of neurons has to be strictly controlled in order for them to be able to make functional columns which will enable the organism to optimally adapt to its environment.

## **Expansion of the Neocortex and Human Brain Evolution**

There is always one species that cannot escape our attention—our own. Numerous studies tried to find human-specific brain features and grasp the notion of “what makes us human”. The development of novel techniques and methods of studying the process of brain development and its underlying molecular mechanisms, together with the insights obtained from comparing different species, opens opportunities to pin-point the traits which might have led to the evolution of the human brain.

Comparative brain transcriptome studies showed that the human genome differs from the chimp genome in only a subset of genes (Caceres et al., 2003; Enard et al., 2002; Khaitovich et al., 2005; Khaitovich et al., 2004). What is becoming more evident is that the differences which led to the emergence of the human brain will be found in the non-protein coding regions of the human genome (King and Wilson, 1975), which have a



**Figure 6. The effects of different neural progenitor lineages on neuron production. (A)** Graphs showing the increase in neuron number from different neural progenitor lineages over the course of 10 cell cycles, with each lineage starting with 1 apical radial glia (aRG). Different colours represent the different neural progenitor lineages as shown in (B), some of which still require experimental validation. The graphs in the left and right panels in (A) illustrate the neuron production from lineages without (left panel) and with (right panel) TAPs, respectively; note the 10-fold difference in ordinate scale. **(B)** Lineages of neural progenitor cells leading to different neuronal outputs; circular arrows, self-renewing progenitors: **(1)**=an aRG divides asymmetrically into a self-renewing aRG and a neuron. **(2)**=an aRG divides asymmetrically into a self-renewing aRG and a BP. The BP divides symmetrically in a self-consuming manner producing two neurons. **(3)**=an aRG divides asymmetrically into a self-renewing aRG and a bRG. The bRG divides asymmetrically into a self-renewing bRG and a neuron. **(4)**=an aRG divides asymmetrically into a self-renewing aRG and a bRG. The bRG divides asymmetrically into a self-renewing bRG and a BP. The BP divides symmetrically in a self-consuming manner producing two neurons. **(5)**=an aRG divides asymmetrically into a self-renewing aRG and a TAP. The TAP divides symmetrically in a proliferative manner (half-circular arrows) giving rise to two TAPs. In their final cell division, each of the TAPs divides symmetrically in a self-consuming manner producing two neurons. **(6)**=an aRG divides asymmetrically into a self-renewing aRG and a bRG. The bRG divides asymmetrically into a self-renewing bRG and a TAP. The TAP divides symmetrically in a proliferative manner (half-circular arrows) giving rise to two TAPs. In their final cell division, each of the TAPs divides symmetrically in a self-consuming manner producing two neurons.

regulatory role. Indeed, some human-specific regions of the non-protein coding genome which show signs of positive selection have already been described (Pollard et al., 2006). Furthermore, signs of positive selection have also been found in promoters of genes involved in neural development and functioning and the regulation of glucose metabolism (Haygood et al., 2007). These changes in glucose metabolism might be connected with the fact that the human brain, being relatively big, has an extreme demand for energy.

The histological comparisons of the human brain with the brains of other primates have shown that, in these terms, the human brain does not fall out of the predicted primate scaled-up brain (Azevedo et al., 2009).

## **CONCLUSIONS AND FUTURE PERSPECTIVES**

In the future, it will be necessary to further explore the regulatory mechanisms and epigenetic modifications which, in interplay with the cell biological features of neural progenitors (spindle orientation, centrosome inheritance, signaling pathways), led to an increase in the production of neurons. All of these forces playing in synchrony with ecological and sociological factors made the human brain a highly complex system. This complexity is reflected in the human susceptibility to diseases of the nervous system (Konopka and Geschwind, 2010). The elucidation of mechanisms underlying the development of the brain will not only satisfy our innate curiosity about our species, but also help in treating numerous diseases which might have their origin in the errors made during divisions of neural progenitors.

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## **ABBREVIATIONS**

|     |                     |
|-----|---------------------|
| APs | Apical progenitors  |
| BPs | Basal progenitors   |
| bRG | Basal radial glia   |
| CSF | Cerebrospinal fluid |
| E   | Embryonic day       |

|      |                                |
|------|--------------------------------|
| INM  | Interkinetic nuclear migration |
| NE   | Neuroepithelial cells,         |
| RG   | Radial glia                    |
| SNPs | Short neural precursors        |
| SVZ  | Subventricular zone            |
| TAPs | Transit amplifying progenitors |
| VZ   | Ventricular zone               |

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# Dynamic Gene Networks in Neural Stem Cell Regulation

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## SUMMARY

Embryonic neural stem cells (NSCs) give rise to various types of neurons, initially deep-layer neurons and then upper-layer neurons, and finally differentiate into astrocytes. During this process, NSCs switch their differentiation competency by changing gene expression profiles regulated by transcription factors. In these cells, expression of the Notch effector *Hes1*, a transcriptional repressor, oscillates with a period of 2–3 hours and *Hes1* oscillations drive the oscillatory expression of the proneural gene *Neurogenin2* (*Ngn2*) and the Notch ligand gene *Delta-like1* (*Dll1*). *Dll1* oscillation leads to the mutual activation of Notch signaling between neighboring cells. After *Hes1* expression is repressed, *Ngn2* is expressed in a sustained manner, promoting neuronal differentiation. Thus, *Ngn2* leads

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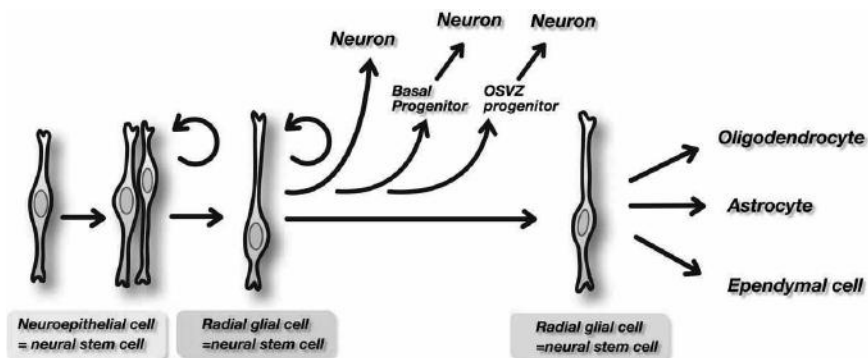
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List of abbreviations after the text.

to the maintenance of NSCs when its expression oscillates but to neuronal differentiation when its expression is sustained. Similarly, not all cells express *Hes1* in an oscillatory manner: cells in boundary regions such as the isthmus express *Hes1* in a sustained manner and these cells are dormant. Thus, *Hes1* controls proliferation and differentiation when its expression oscillates but induces dormancy when its expression is sustained. In the adult brain, NSCs produce neurons that play important roles in brain functions. In the absence of Notch signaling, virtually all NSCs differentiate into neurons and are depleted, indicating that Notch signaling is essential for maintenance of adult NSCs and continuation of adult neurogenesis. While embryonic NSCs are active in proliferation and differentiation, adult NSCs are mostly dormant, although both cells are regulated by Notch signaling. It is possible that dynamics of Notch signaling is different between embryonic and adult NSCs but the exact mechanism remains to be determined.

## INTRODUCTION

In the developing central nervous system (CNS), neural stem cells (NSCs) proliferate in the ventricular zone lining the neural tube. At early embryonic stages, neuro-epithelial cells initially proliferate to expand the NSC pool by symmetric cell divisions (Figure 1). Then, the division mode switches from symmetric to asymmetric by mid-gestation and NSCs initiate neurogenesis



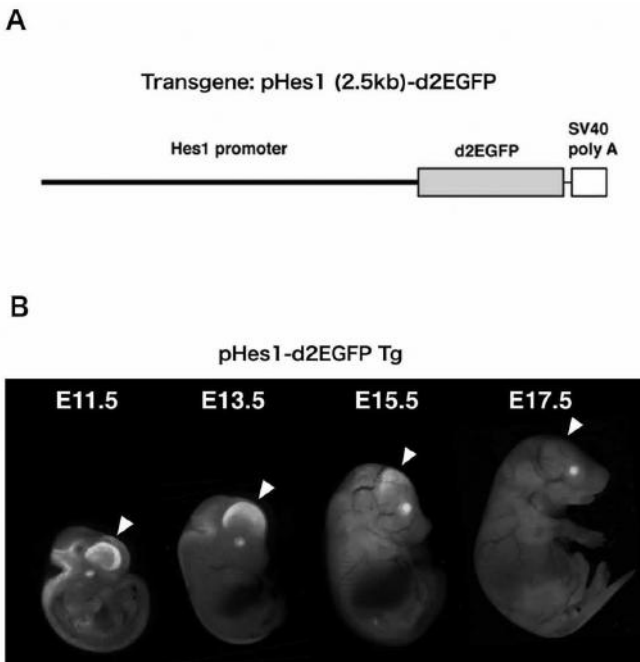
**Figure 1. NSCs temporally alter their morphology and characteristics during cortical development.** During development, NSCs transform from neuroepithelial cells to radial glial cells and finally remain as astrocyte-like cells in the postnatal and adult brain. Neuroepithelial cells initially divide symmetrically to exponentially expand the NSC pool. After the onset of neurogenesis, NSCs adopt a radial glial morphology and sequentially produce basal progenitors and deep layer neurons and then superficial layer neurons by asymmetric, self-renewing divisions. Gliogenesis then supersedes neurogenesis at the late embryonic or perinatal stage, at which time cortical NSCs generate mainly astrocytes and finally transform into astrocytes or ependymal cells, while a subset of NSCs retain their stemness and lurk in the postnatal and adult brain.

by producing one differentiated neuronal daughter and another stem cell daughter (Figure 1) (Chenn and McConnell, 1995; Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2002; Takahashi et al., 1994; Tamamaki et al., 2001). NSCs at this stage are called radial glial cells because they have glial features and radial processes extending apically to the ventricular surface and basally to the pial surface. During the neurogenic stages, radial glial cells sequentially produce deep layer neurons and then superficial layer neurons by asymmetric, self-renewing divisions. Radial glial cells also give rise to basal progenitors, which retract apical and basal processes, migrate into the subventricular zone (SVZ) and divide once or twice to generate more neurons (Figure 1). It was recently found that radial glial cells also give rise to outer SVZ (OSVZ) progenitors, which lose the apical processes reaching the ventricular surface but retain radial fibers reaching the pial surface (Figure 1). OSVZ progenitors undergo asymmetric cell division, which generates another OSVZ progenitor and a neuron (Figure 1) (Fietz et al., 2010; Hansen et al., 2010; Shitamukai et al., 2011). Gliogenesis then supersedes neurogenesis at the late embryonic or perinatal stage and, thereafter, cortical NSCs generate mainly astrocytes and ependymal cells (Figure 1). Only a subset of NSCs adopt the astrocyte-like morphology while retaining their stemness and lurk in the postnatal and adult brain (Alvarez-Buylla et al., 2002; Doetsch et al., 1999; Weissman et al., 2001). Thus, NSCs undergo stepwise differentiation with such alterations in morphology and characteristics in a temporally regulated manner (Temple, 2001) and produce a variety of cell types during cortical development, thereby providing the basis for the complex structure and higher functions of the mammalian neocortex. Unless this transition timing is strictly controlled, the size and shape of the brain and its cellular composition are severely affected.

It has been revealed that *Drosophila* neuroblasts sequentially alter their characteristics over time and express stage-specific transcription factors (Hunchback → Krüppel → Pdm → Castor), and that their progeny marked by the specific gene expression differentiate into neurons with different functions (Isshiki et al., 2001). However, such stage-specific gene expression in NSCs has not yet been elucidated in the mammalian brain. Another important issue is that it takes a long time for NSCs to produce the appropriate number of all cell types. Thus, it is essential to maintain NSCs until the final stages and it has been shown that Notch signaling plays an important role in this process. Here, we review the recent findings about the stage-specific gene expression profiles of NSCs and the role of Notch signaling in their maintenance. In this chapter, neuro-epithelial cells and radial glial cells in the embryonic brain and astrocyte-like stem cells in the adult brain are collectively called NSCs.

## QUANTIFYING GENE EXPRESSION PROFILES OF EMBRYONIC NSCS

*Hes1* and *Hes5*, repressor-type basic helix-loop-helix (bHLH) genes, are expressed within the ventricular zone throughout the developing nervous system, where they function to inhibit neuronal differentiation and to maintain NSCs (Ishibashi et al., 1994; Nakamura et al., 2000; Ohtsuka et al., 1999; Ohtsuka et al., 2001; Tomita et al., 1996). To visualize NSCs, our laboratory has generated transgenic mouse lines in which *Hes* promoters (*pHes*) drive the expression of destabilized enhanced green fluorescent protein (d2GFP), the *pHes1*-d2GFP (Figure 2A) and *pHes5*-d2GFP mice (Ohtsuka et al., 2006). In these mice, GFP was highly expressed in the developing nervous system (Figure 2B). Most GFP+ cells express Pax6 and Nestin, markers for NSCs (Gotz et al., 1998) but negative for the neuronal marker beta-III-tubulin (Tubb3) and the intermediate progenitor cell marker Tbr2 (Eomes) (Englund et al., 2005). Furthermore, these GFP+ cells have the ability to efficiently form neurospheres, suggesting that NSCs are enriched in the GFP+ population.



**Figure 2.** Visualization of NSCs using *pHes1*-d2GFP transgenic mice. (A) Structure of the *pHes1*-d2EGFP transgene (Ohtsuka et al., 2006). (B) GFP expression in *pHes1*-d2EGFP mice at different developmental stages. Arrowheads indicate GFP expression in the dorsal telencephalon. (Adapted from Ohtsuka et al., 2011).

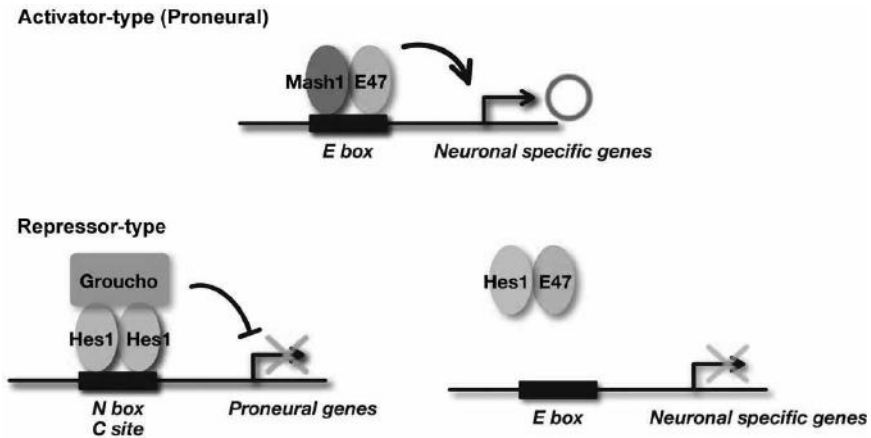
To understand the molecular basis for different competencies of NSCs over time, GFP+ cells were prepared from the developing cortex of *pHes1-d2GFP* mouse embryos at different stages, and gene expression profiling was analyzed (Ohtsuka et al., 2011) (Figure 3A). Gene expression profiling revealed that NSCs dynamically alter their characteristics during cortical development. More than 25,000, of a total of 45,000, genes were detected and thousands of them exhibited more than two-fold changes between two given time points of cortical development. Among them, 92 transcription factors were differentially expressed during the various developmental stages—53 genes being upregulated as development proceeds and 39 genes being downregulated.

Further analyses showed that NSCs in the developing cortex can be categorized into at least four stages by a combination of expression patterns for multiple transcription factors: *Jarid2*, *Trp53*, *Tcf-3* and *-4*, *Klf15*, and *Pippin* (*Csdc2*) with a specific signature of high versus low expression for each according to developmental stage (Figure 3B and C). Most of these transcription factors were found to inhibit neuronal differentiation, suggesting that they play a role in the maintenance of stage-specific NSC (Ohtsuka et al., 2011).

It has been shown that the bHLH genes are key regulators of neuronal fate determination and differentiation during development of the CNS (Bertrand et al., 2002; Kageyama et al., 2007; Ross et al., 2003). There are two types of bHLH factors, the activator type (proneural) such as *Neurogenin* (*Ngn*), *Mash1* (*Ascl1*) and *Math1* (*Atoh1*) and the repressor type such as *Hes1* and *Hes5* (Figure 4). Proneural factors like *Mash1* form heterodimer complexes with another bHLH factor, *Tcf3*(E47), and activate neuronal-specific gene expression, thereby promoting neuronal differentiation. By contrast, repressor-type bHLH factors inhibit neuronal differentiation not only by repressing the expression of proneural factors but also by forming non-functional heterodimer complexes with proneural factors (Bertrand et al., 2002; Kageyama et al., 2007; Ross et al., 2003). Gene profiling analysis showed that some activator-type bHLH genes exhibit dynamic alterations in expression during development. In particular, *Ngn2* and *Math2* (*Neurod6*), which promote neuronal differentiation, are conspicuously upregulated between E11 and E13, in agreement with the onset of extensive neurogenesis (Ma et al., 1996; Shimizu et al., 1995). *Ngn2* and *Math2* expression were maintained at a high level during the neurogenic period until E15 and then declined between E15 and E17 in accordance with the transition from neurogenesis to gliogenesis. Among the repressor-type bHLH genes (*Hes1* and *Hes5*) and *Hes-related* genes (*Hey1* and *Hey2*), only *Hey2* exhibited a significant increase between E11 and E13, while other *Hes* or *Hes-related* genes showed rather stationary expression patterns during development, suggesting that most *Hes* genes are involved in maintenance of NSCs at all stages.



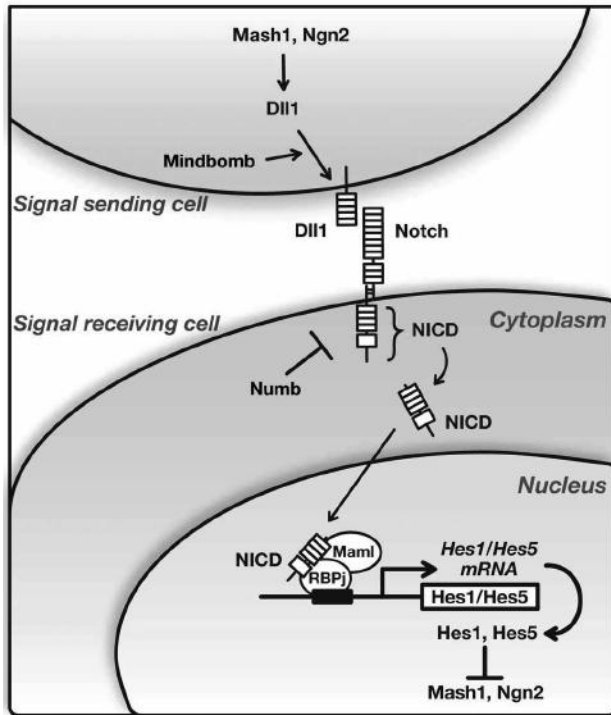




**Figure 4. Two types of bHLH factors.** There are activator-type (proneural) and repressor-type bHLH factors. Activator-type bHLH factors such as Mash1 form heterodimers with another bHLH factor E47(Tcf3) and activate the neuronal differentiation program. By contrast, repressor-type bHLH factors such as Hes1 repress the expression of activator-type bHLH factors and inhibit the DNA-binding activity of activator-type bHLH factors by forming non-functional heterodimers, thereby inhibiting neuronal differentiation and maintaining NSCs.

## NOTCH SIGNALING IN NSCS

As stated above, embryonic NSCs cannot generate all cell types at once but sequentially give rise to different types of cells by gradually changing their competency. Thus, it is very important to maintain NSCs until the final point to generate the proper number of cells and the full diversity of cell types. It has been shown that Notch signaling plays an essential role in maintenance of NSCs. The transmembrane protein Notch is activated by its ligands such as Deltalike1 (Dll1) and Jagged1 (Fortini, 2009; Kopan and Ilagan, 2009; Pierfelice et al., 2011). In differentiating neurons, proneural genes such as *Mash1* and *Ngn2* induce *Dll1*, which activates Notch signaling in neighboring cells (Figure 5). Upon activation of Notch signaling, Notch is cleaved by Adam and then by gamma-secretase, releasing the Notch intracellular domain (NICD) from the transmembrane region. NICD is transferred to the nucleus and forms a complex with the DNA-binding protein Rbpj and the transcriptional co-activator Mastermind-like (Mam1) (Figure 5). This NICD-Rbpj-Mam1 complex induces the repressor-type bHLH genes such as *Hes1* and *Hes5*, which repress proneural gene expression. As a result, the latter cell remains an NSC. Thus, differentiating neurons inhibit neighboring cells from differentiating into the same cell type—this process is called lateral inhibition.



**Figure 5. The core pathway of Notch signaling.** In differentiating neurons, proneural genes such as *Mash1* and *Ngn2* induce the expression of the Notch ligand *Dll1*, which in turn activates Notch signaling in neighboring cells. Upon activation of Notch, the Notch intracellular domain (NICD) is released from the transmembrane portion and transferred to the nucleus, where it forms a complex with the DNA-binding protein Rbpj and the co-activator Maml. The NICD-Rbpj-Maml complex induces the expression of *Hes1* and *Hes5*, which then repress the expression of proneural genes and *Dll1*, thereby maintaining NSCs.

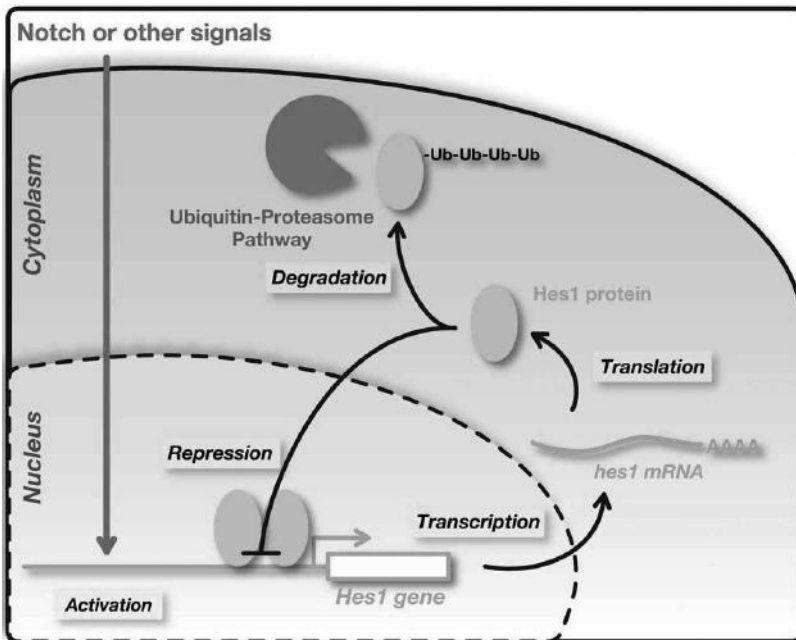
Inactivation of *Hes1* and *Hes5* upregulates proneural gene expression, accelerating neuronal differentiation and depleting NSCs (Hatakeyama et al., 2004). Similarly, inactivation of Rbpj accelerates neuronal differentiation and depletes NSCs (Imayoshi et al., 2010). Conversely, overexpression of NICD maintains NSCs but cannot do so in the absence of *Hes1* and *Hes5* (Ohtsuka et al., 1999). Thus, the NICD-Rbpj-Hes1/5 pathway regulates the maintenance of NSCs by repressing proneural gene expression.

Notch signaling plays an important role in asymmetric cell division. During this mode of cell division, Numb, which is known to inhibit Notch signaling (Rhyu et al., 1994), is asymmetrically distributed into one of two daughter cells. Numb-positive cells become negative for *Hes1* and

differentiate into neurons while Numb-negative cells express Hes1 and remain NSCs (Ohtsuka et al., 2006; Shen et al., 2002). Thus, Numb-dependent regulation of Notch signaling is important for neuronal differentiation.

## Dynamic Expression of Notch Signaling Molecules

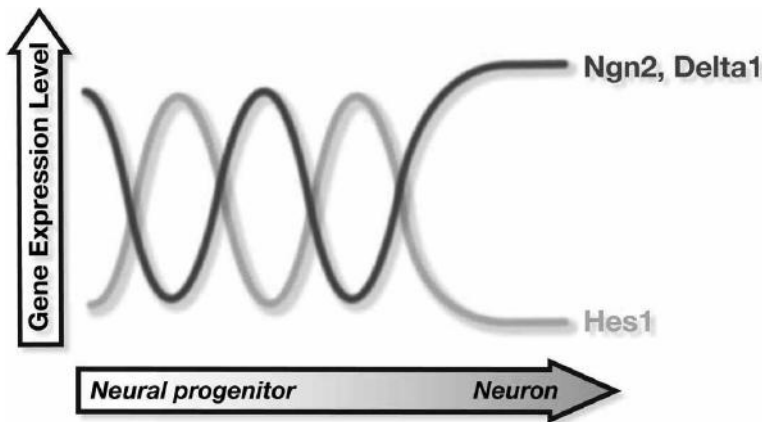
Hes1 expression is variable in NSCs. It has been shown that Hes1 expression oscillates in a period of about two hours in many cell types such as fibroblasts (Hirata et al., 2002). This oscillatory expression is regulated by negative feedback (Figure 6). Activation of Hes1 promoter leads to synthesis of Hes1 protein, which represses its own expression by directly binding to the Hes1 promoter. Repression of Hes1 promoter leads to disappearance of Hes1 protein, because it is extremely unstable; the disappearance of Hes1 protein relieves negative repression and allows the next round of synthesis. In this way, Hes1 expression autonomously oscillates. Time-lapse imaging analysis using the Hes1 promoter-driven destabilized luciferase reporter showed that Hes1 expression oscillates in mouse NSCs in a period of



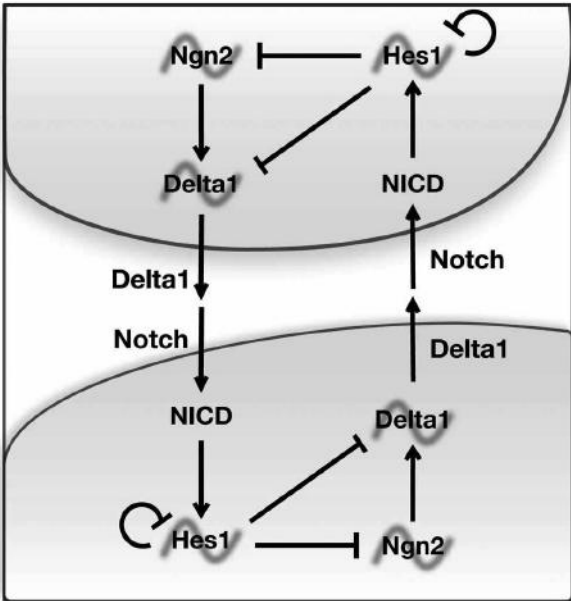
**Figure 6. Oscillatory expression of Hes1.** Hes1 expression oscillates with a period of 2–3 hours in NSCs. When the Hes1 promoter is activated, Hes1 protein is produced, but it represses its own expression by directly binding to the Hes1 promoter. This negative feedback leads to the disappearance of Hes1 mRNA and Hes1 protein, both extremely unstable, allowing the next round of expression. In this way, Hes1 autonomously starts oscillatory expression. Ub indicates ubiquitination for Hes1 protein degradation.

about 2–3 hours (Figure 7) (Shimojo et al., 2008). It was shown that Hes1 oscillation is important for efficient cell proliferation of fibroblasts because both sustained (non-oscillatory) expression and loss of expression of Hes1 slows cell proliferation (Yoshiura et al., 2007). Similarly, sustained Hes1 expression inhibits proliferation of NSCs (Baek et al., 2006), suggesting that Hes1 oscillation is required for efficient cell proliferation. The developing nervous system is partitioned into many compartments by boundaries such as the isthmus and the right and left halves by the roof and the floor plate. Cells in these boundaries usually proliferate slowly or remain quiescent. It was found that Hes1 expression is sustained in these boundary cells, suggesting that sustained Hes1 expression regulates slow proliferation or quiescent state of boundary cells (Baek et al., 2006). The mechanism for sustained Hes1 expression in boundary cells remains to be determined.

The lateral inhibition mechanism suggests that maintenance of NSCs requires differentiating neurons in the next neighbor because such neurons express Notch ligands. This raises a question of how NSCs are maintained at early stages of development before neurons are born. It was found that proneural genes and Notch ligands are expressed in a salt-and-pepper pattern by NSCs at early stages. In these cells, proneural gene and Notch ligand gene expression shows an inverse correlation with Hes1 expression (Baek et al., 2006). Because Hes1 expression oscillates, this observation suggests that proneural gene and Notch ligand gene expression also oscillates in NSCs. Time-lapse imaging analysis demonstrated that expression of the proneural gene *Ngn2* and the Notch ligand *Dll1* oscillates in NSCs (Figure 7) (Shimojo et al., 2008). Because Hes1 represses *Ngn2* and *Dll1* expression by directly binding to the promoters, it is likely that Hes1 oscillation leads to *Ngn2* and *Dll1* oscillation in NSCs. In support of this idea, in differentiating neurons where Hes1 expression is repressed, *Ngn2* and *Dll1* expression becomes sustained (Shimojo et al., 2008). From these results, it seems that the oscillatory versus sustained expression mode of proneural genes is important for the outcome: when the expression is sustained, proneural genes induce neuronal differentiation whereas when the expression is oscillatory, it regulates the maintenance of NSCs. Many of the downstream genes are expressed when proneural genes are sustained but not when the proneural genes are oscillating. It is likely that when proneural gene expression oscillates, only genes that respond quickly, such as *Dll1*, are expressed in an oscillatory manner. Thus, proneural gene oscillation leads to mutual activation of Notch signaling by inducing *Dll1* oscillation and maintenance of NSCs without induction of neuronal differentiation (Figure 8). This raises the possibility that oscillation is an essential mechanism to activate Notch signaling without the help of any neurons and to keep a group of cells undifferentiated.



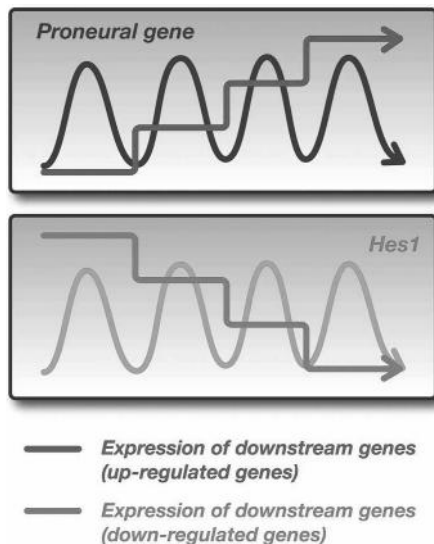
**Figure 7. Dynamic expression of proneural and Notch ligand genes in NSCs.** In NSCs, Hes1 oscillation drives *Ngn2* and *Dll1* oscillations. When the expression is oscillating, the proneural gene *Ngn2* cannot induce neuronal differentiation. Instead, *Ngn2* induces *Dll1* oscillation and maintains NSCs. When Hes1 expression disappears, *Ngn2* and *Dll1* expression becomes sustained. When the expression is sustained, *Ngn2* induces neuronal differentiation. Thus, the expression mode (oscillatory versus sustained) of proneural genes may be important for the outcome.



**Figure 8. Maintenance of NSCs by the mutual activation of Notch signaling.** *Ngn2* oscillation induces *Dll1* oscillation, which leads to mutual activation of Notch signaling between NSCs. Thus, *Ngn2* and *Dll1* oscillations may be advantageous for the maintenance of NSCs during early stages of development without *Dll1* stimulation from neurons, and a salt-and-pepper pattern induced by lateral inhibition is just a snapshot of oscillatory expression.

These results suggest that a salt-and-pepper expression pattern of proneural and Notch ligand genes in NSCs may be a snapshot of oscillatory expression. It is generally believed that when a salt-and-pepper expression pattern exists, cells positive for proneural and Notch ligand gene expression are already selected to become neurons first while negative cells remain NSCs. However, positive cells could become negative while negative cells could become positive a few hours later, suggesting that both positive and negative cells may still remain NSCs and that positive cells do not necessarily initiate neuronal differentiation first (Kageyama et al., 2008).

NSCs change their competency over time but the exact mechanism is unknown. Isolated single NSCs cultured *in vitro* can autonomously change their competency like *in vivo*, suggesting that this feature is not directed by environmental conditions but is programmed in NSCs (Qian et al., 2000). Oscillatory expression of Hes1 and proneural genes could lead to gradual changes of downstream gene expression, which could cause differential gene expression profiles, thereby contributing to different competencies of NSCs (Figure 9). If the products of downstream genes are stable, they might accumulate in a stepwise manner when their expression is activated by oscillating proneural genes (Figure 9). Thus, oscillatory expression of Hes1 and proneural genes could also contribute to the heterogeneity of NSCs. It has been shown that Hes1 oscillation plays an important role in the heterogeneity of embryonic stem (ES) cells. ES cells are known to



**Figure 9. Possible downstream gene expression patterns regulated by oscillating proneural and Hes1 genes.** Oscillatory expression of proneural and Hes1 genes could lead to gradual changes of downstream gene expression, which could make different gene expression profiles, thereby contributing to different competencies of NSCs.

respond differently to the same signals: for example, even under the neural induction condition, not all ES cells differentiate into neural cells. It was revealed that in mouse ES cells, *Hes1* expression oscillates in a period of about 3–5 hours (Kobayashi et al., 2009). Interestingly, ES cells expressing *Hes1* at a low level tend to differentiate into neural cells, whereas those expressing *Hes1* at a high level tend to differentiate into mesodermal cells (Kobayashi and Kageyama, 2010; Kobayashi et al., 2009). Furthermore, *Hes1*-null ES cells tend to differentiate into neural cells more uniformly at an earlier timing. These results suggest that *Hes1* oscillations contribute to the heterogeneity in differentiation competency of ES cells. Different *Hes1* expression levels might also generate different differentiation responses of NSCs to the same signals, although it remains to be determined whether *Hes1* oscillation produces such heterogeneity of NSCs.

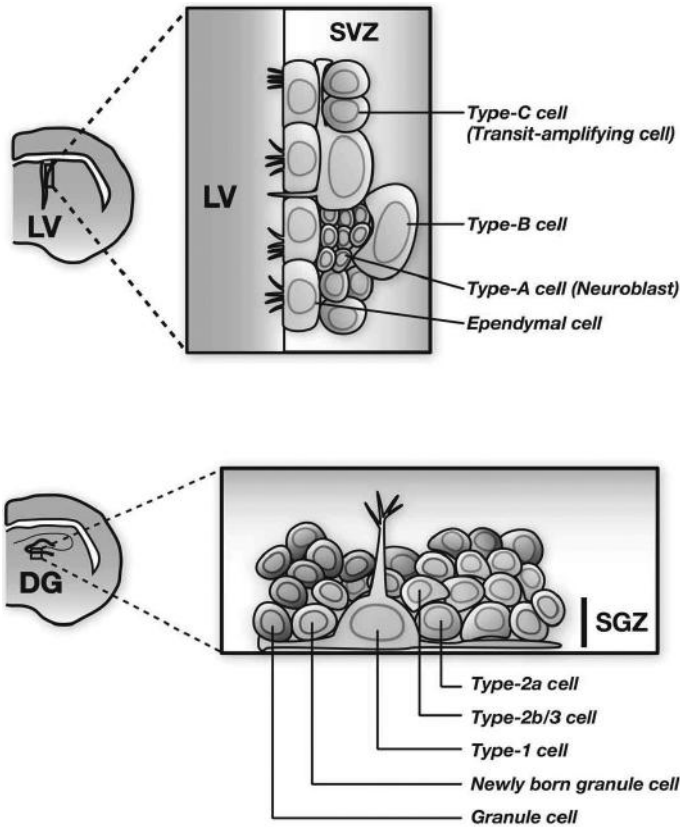
### **Notch Signaling in Basal Progenitors and OSVZ Progenitors**

Whereas maintenance of basal progenitors depends on Notch signaling, these cells express less *Hes1* and *Hes5* than radial glial cells, suggesting that *Rbpj* is not active in basal progenitors (Kawaguchi et al., 2008; Mizutani et al., 2007). Furthermore, knock-down of *Rbpj* converts NSCs into basal progenitors, suggesting that *Rbpj*-dependent Notch signaling regulates NSCs whereas *Rbpj*-independent Notch signaling regulates basal progenitors. Another feature for basal progenitors is that they express *Tbr2*, which is induced by *Ngn2*. It is likely that *Tbr2* expression is inhibited by *Rbpj*-dependent Notch signaling in NSCs but not by *Rbpj*-independent Notch signaling in basal progenitors. However, the precise mechanism of *Rbpj*-independent Notch signaling remains to be determined.

In contrast to basal progenitors, OSVZ progenitors express *Hes1*, suggesting that Notch signaling is activated in these cells (Hansen et al., 2010). Interestingly, their daughter cells (OSVZ progenitors and neurons) maintain contact with each other for several hours, and neurons seem to express Notch ligands and activate Notch signaling in their sibling OSVZ progenitors (Shitamukai et al., 2011). These observations suggest that asymmetric cell division is required to activate Notch signaling in OSVZ progenitors by their sibling neurons. It remains to be determined what types of neurons are differentiated from OSVZ progenitors.

### **ADULT NEUROGENESIS AND NSCS**

Neurogenesis occurs actively in two regions of the adult brain: the SVZ of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Figure 10) (Ming and Song, 2005; Zhao et al., 2008). In



**Figure 10. NSC and neurogenesis in the adult brain.** NSCs are present in two regions of the adult brain, the subventricular zone (SVZ) of the lateral ventricle (LV) and the subgranular zone (SGZ) of the hippocampal dentate gyrus. These NSCs are mostly quiescent but occasionally divide to give rise to transit-amplifying cells, which proliferate and generate neurons. Neurons born in the SVZ migrate via the rostral migratory stream into the olfactory bulb, while neurons born in the SGZ migrate into the hippocampal dentate gyrus.

*Color image of this figure appears in the color plate section at the end of the book.*

these regions, there are many NSCs. These cells are mostly quiescent but occasionally divide to give rise to transit-amplifying cells which proliferate and generate many neurons. Neurons born in the adult SVZ migrate via the rostral migratory stream into the olfactory bulb (Figure 10), while neurons born in the SGZ migrate into the hippocampal dentate gyrus (Figure 10). Neurogenesis in the dentate gyrus is important for learning and memory while neurogenesis in the olfactory bulb is required for innately programmed olfactory responses such as sexual and maternal behaviors (Imayoshi et al., 2008; Kee et al., 2007; Kempermann, 2008; Lagace et al., 2010; Sakamoto et al., 2011; Zhao et al., 2008). Adult NSCs express Hes1



and *Hes5*, suggesting that Notch signaling is required for their maintenance (Imayoshi et al., 2010). Indeed, inactivation of Notch signaling molecules like *Rbpj* in the adult NSCs leads to acceleration of neuronal differentiation and depletion of these cells (Ables et al., 2010; Breunig et al., 2007; Ehm et al., 2010; Imayoshi et al., 2010; Nyfeler et al., 2005; Veeraraghavalu et al., 2010). In the absence of *Rbpj*, neurogenesis is enhanced transiently but ceases within 3 months (Imayoshi et al., 2010). Thus, Notch signaling is essential for maintenance of adult NSCs and continuation of adult neurogenesis.

Although Notch signaling is involved in maintenance of both embryonic and adult NSCs, the features of these cells are very different. For example, embryonic NSCs proliferate extensively and sequentially give rise to many distinct cell types while adult NSCs are mostly quiescent and generate a few subsets of neurons. The exact mechanism for these differences are unknown, but it is possible that *Hes1* expression mode could be different: *Hes1* expression may not be dynamic in adult NSCs while it is oscillating in embryonic NSCs. Further analysis on *Hes1* expression dynamics in adult NSCs will be required to understand the mechanism for differences between embryonic and adult NSCs.

## CONCLUSION

Embryonic NSCs change their competency over time and it was revealed that the gene expression profiles including many transcription factor genes in NSCs are also changing, suggesting that expression profiles provide the basis for different competency of NSCs. However, expression of the Notch effector gene *Hes1* and *Hes5* in NSCs is not changed, suggesting that Notch signaling is involved in maintenance of NSCs at all stages. *Hes1* expression oscillates in NSCs but is sustained in boundary cells. Importantly, oscillatory versus sustained expression of *Hes1* leads to different outcomes in NSCs. When its expression oscillates, NSCs proliferate actively and differentiate into neurons. By contrast, when its expression is sustained, NSCs become dormant. Similarly, oscillatory versus sustained expression of the proneural gene *Ngn2* leads to different outcomes. When its expression is sustained, NSCs differentiate into neurons. By contrast, when its expression oscillates, NSCs remain undifferentiated. Thus, not just the expression but also the dynamics of these genes are very important for the outcomes. It is also possible that oscillating genes may be involved in altering the gene expression profiles and changing the competency of embryonic NSCs over time, although the precise mechanism remains to be determined. In the adult brain, NSCs are maintained by Notch signaling and produce neurons, which play an important role in brain functions. However, adult NSCs are mostly

dormant in proliferation and differentiation. It is possible that dynamics of Notch signaling molecules are different between embryonic and adult NSCs, but the exact mechanism remains to be determined.

## Abbreviations

|       |                                                                        |
|-------|------------------------------------------------------------------------|
| Adam  | A disintegrin and metalloproteinase domain                             |
| Atoh  | Atonal homolog                                                         |
| bHLH  | Basic helix-loop-helix                                                 |
| Csdc  | Cold shock domain containing C                                         |
| Dll   | delta-like                                                             |
| Hes   | Hairy and enhancer of split                                            |
| Hey   | Hairy/enhancer-of-split related with YRPW motif                        |
| Hmga2 | High mobility group AT-hook                                            |
| Jarid | Jumonji, AT rich interactive domain                                    |
| Klf15 | Kruppel-like factor 15                                                 |
| Maml  | mastermind like                                                        |
| Ngn   | Neurogenin                                                             |
| NICD  | Notch intracellular domain                                             |
| Numb  | numb gene homolog                                                      |
| OSVZ  | Outer sub-ventricular zone                                             |
| Rbpj  | recombination signal binding protein for immunoglobulin kappa J region |
| SGZ   | subgranular zone                                                       |
| SVZ   | Sub-ventricular zone                                                   |
| Tcf   | T-cell factor                                                          |
| Trp   | Transformation related protein                                         |

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# Stem Cells in the Development, Regeneration and Repair of the Retina

*Thomas A. Reh*

## SUMMARY

The development of the eye has long fascinated embryologists. The great classical developmental biologist, Hans Spemann, studied lens induction before his pioneering investigations of the organizer and neural induction. In addition, it was discovered in the late 1700's that parts of the eye regenerate in amphibians. This fascination is likely in part due to the fact that humans are such highly visual animals. Moreover, the loss of sight is probably the most difficult sensory deficit to adjust to. In recent years, however, the understanding of the development and regeneration in this system has reached a new level. Many of the principal molecular systems have been identified. Nevertheless, we are still lacking a coherent model of the mechanisms that control the patterning of the ocular domains during early development and the factors that control cell diversity in the developing retina, despite years of effort. The relatively new emphasis on

the development of protocols to direct embryonic stem cells to eye and retinal fates, for their ultimate use in cell therapy for retinal degenerative diseases, has provided new impetus to better understand the molecular mechanisms of retinal development. This review will focus on three related areas-retinal development, regeneration and stem cells-with an emphasis on new findings, particularly in the mammalian retina, where translational work can have a direct impact on human disease.

## INTRODUCTION

### Early Events in Eye Development

The earliest that the eye forming cells of the embryo can be recognized is in the eye field, a region of the anterior neural plate that becomes committed to develop into several different tissues of the eye, including the neural retina, ciliary epithelium and pigmented epithelium. Transplantation experiments and fate mapping carried out nearly 100 years ago demonstrated that the cells of this region are committed to forming ocular tissues soon after gastrulation. Initially, the eye-field is a single domain that extends across the midline; however, it is split into two domains by midline signaling of sonic hedgehog (Shh) released by the prechordal mesoderm (Li et al., 1997). Since the early 1990's, investigators began to identify transcription factors that were predominantly, or exclusively, expressed in the presumptive eye-field. The "eye field transcription factors" (EFTFs) include the members of the paired-homeodomain family Pax6, Rx, Six3, Lhx2 and Optx2 (Six6) (see Zuber, 2010 for review). In addition to their eye-field expression, the EFTFs are in many cases both necessary and sufficient for much of eye development with mutations leading to anophthalmia (no eye) or microphthalmia (small eyes) (Bernier et al., 2000; Callaerts et al., 1997; Carl et al., 2002; Chow et al., 1999; Hill et al., 1991; Mathers et al., 1997; Porter et al., 1997; Zuber et al., 2003).

The first EFTF that was discovered was Pax6 (Gehring, 1996). Pax6 is a member of the paired box homeodomain genes and was identified in humans from mutations that cause aniridia, a defect in the formation of the iris. Around the same time, Gehring's group found that ectopic expression of the *Drosophila* homolog to Pax6, *eyeless*, induced eyes to form in other regions of the animal, like the antenna (Halder et al., 1995). The remarkable result of a conserved molecular pathway controlling eye development in species as distant as humans and *Drosophila* led to the proposal that Pax6 is the master regulator of eye development in all animals. Since these discoveries, studies of Pax6 in many other species have shown that indeed there is a high degree of conservation in many parts of the eye-forming molecular machinery; for example, deletions or loss of function mutations

in Pax6 result in anophthalmia in all mammals, including humans, and in these cases eye development stops prior to the formation of the optic cup (Matsuo et al., 1995).

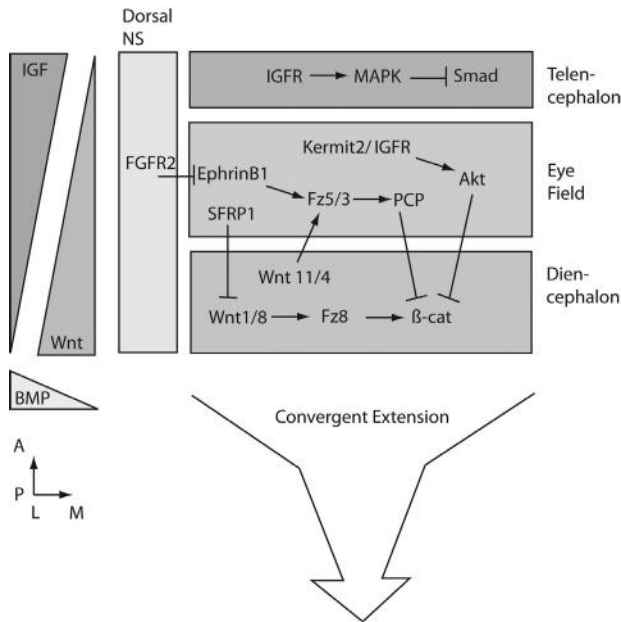
Despite the importance of Pax6, it was soon realized that there are several other transcription factors involved in early eye development. Retinal homeobox, or Rx/Rax, is among the earliest of the EFTFs to be expressed in the eye field. Targeted deletion of the Rx gene in mice results in the failure of eye development even earlier than what is observed in Pax6 deletions (Mathers et al., 1997). The Rx knockout mice lack expression of Pax6, whereas Pax6 mutant mouse embryos still express Rx (Zhang et al., 2000), suggesting that Rx is upstream of Pax6 in the pathway (Zhang et al., 2000). However, over-expression of these and other EFTFs reveal a more complex, non-linear pathway. In *Xenopus*, over-expression of Pax6 induces ectopic eyes and also induces Rx expression (see Zuber, 2010 for review).

Several other transcription factors are expressed in the eye field along with Rx and Pax6. One of these, Lhx2, is a member of the Lim-homeodomain gene family (Porter et al., 1997). In mice with a deletion of Lhx2, eye formation stalls at the optic vesicle stage; the optic cup and lens fail to develop (Porter et al., 1997). Nevertheless, Pax6 is expressed in the optic vesicle in Lhx2 knockout mice, indicating that Lhx2 lies downstream of Pax6. However, over-expression of Lhx2 in *Xenopus* embryo can induce ectopic retinal tissue and activate Pax6 and Rx. Again, the existence of a network of interacting factors is more consistent with the data than a simple linear pathway (Zuber et al., 2003). Two members of the Six-Homeodomain family of genes, Six3 and Six6 (Optx2) are also expressed in the presumptive eye field around the same time as Pax6 (Bovolenta et al., 1998; Jean et al., 1999; Loosli et al., 1999; Oliver et al., 1995; Toy et al., 1998). Inactivation of Six3 leads to anophthalmia and forebrain agenesis (Carl et al., 2002), while over-expression results in multiple eye-like structures (Kobayashi et al., 1998; Loosli et al., 1999). Over-expression of Six6 in *Xenopus* embryos causes a large expansion of the retinal domain (Bernier et al., 2000; Zuber et al., 1999). Another critical transcription factor in eye development is the paired-homeodomain transcription factor, Otx2 (a member of the orthodenticle family) (Simeone, 1998). While not specifically defined as an EFTF, Otx2 has several roles in eye development. Even before the other EFTFs are expressed, Otx2 is expressed in the anterior neural plate, including the presumptive eye fields, prior to the other EFTFs. Deletion of Otx2 in mice leads to the lack of eye fields and any structures anterior to rhombomere 3 (Matsuo et al., 1995). When the other EFTFs begin to be expressed, Otx2 is down-regulated in the eye field; however, Otx2 expression persists in the presumptive pigment epithelium, and ultimately is re-expressed in photoreceptors and bipolar cells (Bovolenta et al., 1997).



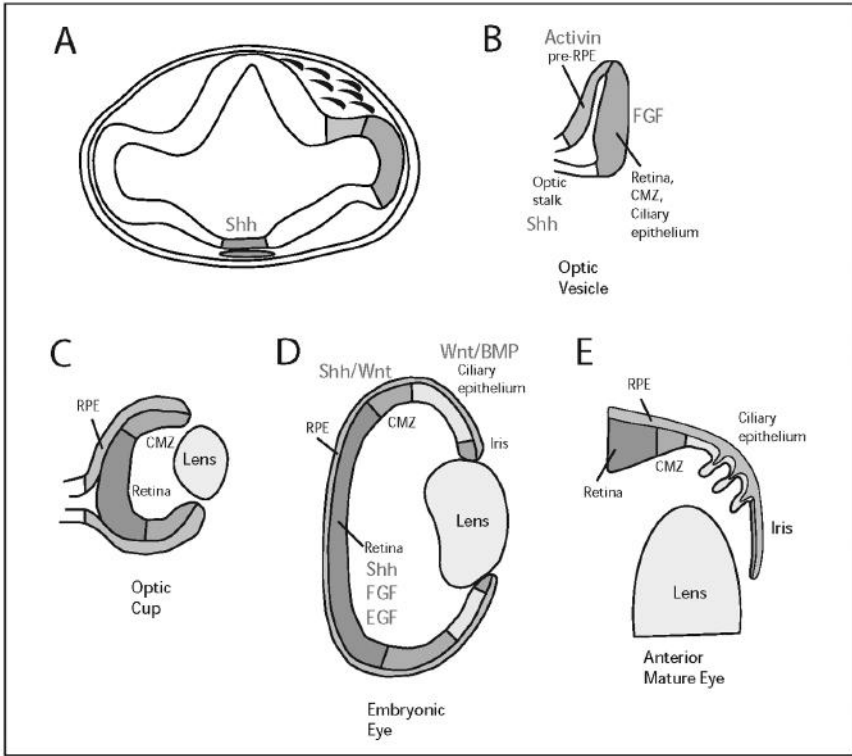
The coordinated expression of the EFTFs in the developing eye field is a striking example of patterning in the neural plate and has led several investigators to study the factors that are responsible for controlling the expression of the EFTFs and thereby defining the eye field. In the last five years this question has generated considerable attention (Esteve and Bovolenta, 2006). One of the key factors in the development of anterior structures is Igf (insulin-like growth factor). Igf and its receptors are highly expressed in the anterior neural plate (Richard-Parpaillon et al., 2002). Over-activation of the Igf pathway in *Xenopus* embryos leads to an expansion of anterior fates, including ectopic eyes (Eivers et al., 2004; Pera et al., 2001; Richard-Parpaillon et al., 2002). Moreover, differences in downstream components of the Igf pathway further refine the eye field (Wu et al., 2006). Differential activation of the Mapk pathway and the PI3K/Akt pathway may underlie the distinction between the eye field and the telencephalon. *Kermit2*, which potentiates the Akt pathway specifically, promotes EFTFs when over-expressed (Wu et al., 2006) whereas inhibition of *Kermit2* blocks eye formation. Another developmental signaling pathway, the Wnt pathway, is also an important regulator of the eye field. Several components of the Wnt pathway, ligands, frizzled receptors and downstream signaling components are expressed in or around the domain of EFTF expression. It appears from a variety of experiments that antagonism of canonical Wnt signaling is required for eye field formation. Wnt/beta-catenin signaling suppresses the EFTFs (Cavodeassi et al., 2005), whereas activation of the non-canonical Wnt pathway, which suppresses the beta-catenin canonical signal, promotes EFTF expression (Cavodeassi et al., 2005; Maurus et al., 2005). Fz5, a receptor associated with non-cannonical signaling, is expressed in the eye field in mice (Liu and Nathans, 2008) and deletion of Fz5 leads to a reduced eye field/optic vesicle. The Wnt inhibitor *Sfrp1* is also expressed in the eye field and knock-down of this gene also leads to a reduction in the expression domain of the EFTFs (Esteve and Bovolenta, 2006). These results fit nicely with a proposed mechanism for Igf in eye field formation. Over-expression of Igf1 inhibits canonical Wnt signaling and rescues eye formation in Wnt8 expressing embryos (Richard-Parpaillon et al., 2002). These studies support a model in which canonical Wnt signaling is necessary for the patterning of the posterior diencephalic domain whereas the inhibition of this pathway promotes EFTF expression (Figure 1).

Soon after the formation of the eye fields, the cells that comprise them undergo a series of complex morphogenic events (see Fuhrmann, 2010 for review). The single eye field is “split” into two domains by inhibition of the midline expression of EFTFs by Shh derived from the prechordal mesoderm (Adelmann, 1936; Li et al., 1997). A similar function for Shh is likely to be true for mammals as well since Shh knockout mice have a single “cyclopean” eye field (Chiang et al., 1996). The next stage of eye development involves



**Figure 1. Patterning factors in the developing eye.** Gradients of morphogenic factors that are important in setting up the eye field (left) are depicted with low levels of Bmp and canonical Wnt signaling and, conversely, high levels of Igf signaling promoting eye field transcription factor expression. The three forebrain domains of the telencephalon, eye field and posterior diencephalon are shown (right) with the specific patterning factors and signaling molecules indicated in the domains where they are expressed and/or active.

the evagination of the eye field cells to form bulges on either side of the midline, the optic vesicles, which go on to subsequently invaginate into cup-like structures called the optic cups (Figure 2). The inner part of the cup will form the neural retina, while the outer layer of the cup will give rise to the pigmented epithelium. At the rim of the cup, the iris and ciliary epithelium form two layered structures, with an inner, non-pigmented layer apposed to an outer, pigmented layer. The mechanisms that pattern the different domains of the optic vesicle and cup are not fully understood, but several factors are thought to be important for their specification and differentiation (for review see Fuhrmann, 2010). FGF signaling promotes neural retina formation in developing chick embryo optic vesicle, while BMP/activin, Shh and Wnt promote pigmented epithelial (RPE) development (Dakubo et al., 2008; Fuhrmann et al., 2000; Fuhrmann et al., 2009; Fujimura et al., 2009; Grocott et al., 2011; Muller et al., 2007; Picker and Brand, 2005; Pittack et al., 1997; Westenskow et al., 2009; Westenskow et al., 2010). The source of these signals is not entirely clear. In the chick embryo, there is evidence that BMP/activin signaling can arise from the extraocular neural crest derived mesenchyme (Fuhrmann et al., 2000; Grocott et al., 2011; Muller et al., 2007).



**Figure 2. Morphogenesis during eye development.** (A) The eye fields are split into two by midline sonic hedgehog signaling (Shh). (B) Several factors that are present in either the surrounding tissues or the adjacent regions of the neuroepithelium pattern the domains of the optic vesicle. (C) The optic vesicle transforms into a cup-shaped structure as the lens forms from the adjacent epithelium. (D) As the optic cup expands, the various domains of the mature eye are patterned. (E) The anterior part of the mature eye in a non-mammalian vertebrate is shown to represent the relationship among the ciliary epithelium and the retina and the stem cell zone known as the CMZ (see text for details; B-E, modified from Lamba et al., 2008).

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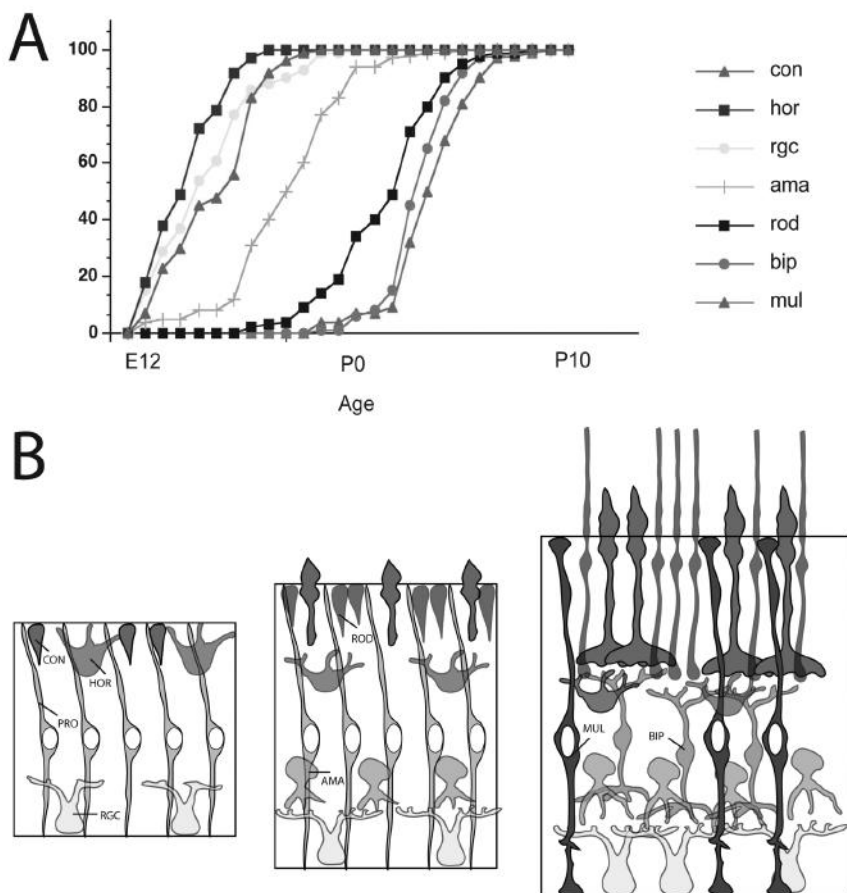
On the other hand, the Shh and Wnt signals that promote RPE development may arise from the adjacent neuroepithelium (e.g., Eiraku et al., 2011). It is likely, therefore, that these partly redundant signaling systems can arise from several sources. The anterior part of the optic cup gives rise to the ciliary epithelium and iris. Less is known about the details of patterning in these tissues, but both Wnt (particularly Wnt2b) and BMP have been shown to be important in this process (see Fuhrmann, 2010 for review).

## NEURAL RETINAL PROGENITORS

### Intrinsic Regulators of Cell Production

Although there is considerable interest in understanding the development of the various types of tissues derived of the optic cup, this review will focus on the neural retina. Like neural progenitors elsewhere in the CNS, the cells of the optic cup have a simple bipolar morphology. In addition, these progenitor cells span the width of the neuroepithelium, but their nuclei move to more vitreal positions when in S-phase and undergo mitotic divisions at the scleral (ventricular) surface. The birthdating studies of Sidman (Sidman, 1959) first demonstrated that the different types of retinal neurons are generated in a sequence: ganglion cells, cone photoreceptors, amacrine cells and horizontal cells generated during the first half of retinogenesis, while most rod photoreceptors, bipolar cells and Müller glia generated in the latter half of retinogenesis. In this way the complex structure of the retina is built up over development (Figure 3). Although the progenitors appear morphologically similar, they have considerable heterogeneity in gene expression, e.g. (Brzezinski et al., 2011). Lineage tracing of the progeny of single progenitor cells has shown that many of the progenitors in the early embryonic retina can produce all the different types of retinal neurons, as well as Muller glia, and therefore the cells are collectively known as “multipotent progenitors” (Holt et al., 1988; Turner and Cepko, 1987); however, more recent analysis of specific populations of progenitors reveals that many of these cells generate only a subset of the total diversity of retinal neurons (Brzezinski et al., 2011; see below).

How do the retinal progenitors generate the diverse types of neurons during retinogenesis? As noted above, there is a conserved birth order in the production of the different types of neurons. Forced differentiation of progenitors at early stages of retinal development causes them to adopt early fates; e.g., ganglion cells, whereas inducing their differentiation at late stages of development causes them to adopt “late” fates, such as rod photoreceptors or Muller glia (Reh and Kljavin, 1989; Watanabe and Raff, 1990). These results, and others, have led to the hypothesis that retinal progenitor cells undergo a progressive change during development that constrains them to a smaller range of fates (Brzezinski et al., 2010; Reh and Cagan, 1994). These results further suggest that some sort of “molecular clock”, intrinsic to the progenitors, keeps track of the developmental stage. Alternatively, the progenitor cells may be competent to generate all retinal cell types throughout the period of retinogenesis, but changes in the microenvironment direct the cells to develop as early or late fates (Reh, 1992; Watanabe and Raff, 1992). Both the microenvironment and intrinsic state of the progenitor cell can influence the types of cells they generate (see Brzezinski et al., 2010 for recent review).



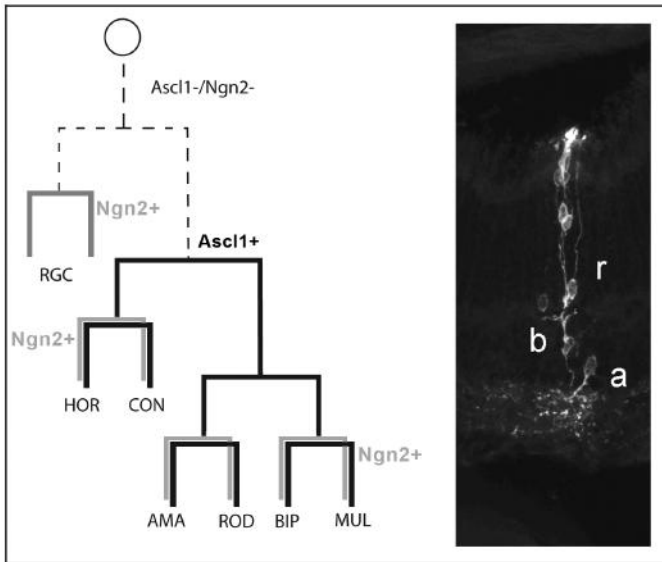
**Figure 3. Sequential development of retinal cells.** (A) Birthdating studies of retinogenesis, showing the sequence in generation of the different types of neurons. CON=cones, HOR=horizontal cells, RGC=ganglion cells, AMA=amacrine cells, ROD=rod photoreceptors, BIP=bipolar cells, MUL=Müller glia. (B) The progenitor cells (light blue) span the retina at early developmental stages and only a few types of neurons have been generated (RGC, CON, HOR). As development proceeds (middle), new cells are added into the existing structure and the retina becomes thicker with amacrine cells (AMA) and rods (ROD). In the last stage of development, bipolar cells and Müller glia are added (right panel).

*Color image of this figure appears in the color plate section at the end of the book.*

The factors that control the generation of the various types of retinal neurons have been the subject of much investigation for many years and a model of cell fate determination in this tissue has emerged, though it is by no means complete. As noted above, early studies indicated that progenitors were not all identical, and over the period of histogenesis they change from an “early” to “late” state of cell fate generation; more recently

this has been correlated with changes in gene expression. While most, if not all, progenitors express the eye field transcription factors, Pax6, Rx, Lhx2, Six3, there is considerable diversity in their expression of other transcription factors (Akagi et al., 2004; Hatakeyama et al., 2001). Several members of the proneural bHLH family of transcription factors are expressed in the retinal progenitors, but they are not uniformly expressed in all retinal progenitors. The bHLH transcription factor Ascl1 (also known as Mash1 or Cash1) is not expressed in the retinal progenitors at eye cup stages (Brzezinski et al., 2011; Jasoni and Reh, 1996; Jasoni et al., 1994), but is expressed by the majority of progenitors at late stages of retinogenesis. Neurog2 (also known as Ngn2) is also expressed in only a subset of progenitors in the retina (Marquardt et al., 2001; Nelson and Reh, 2008). Recent analysis of three of these factors, Ascl1, Neurog2 and a related bHLH transcription factor, Olig2, shows that various combinations of these three bHLH transcription factors exist in subpopulations of retinal progenitors (Brzezinski et al., 2011). Combining the expression data with inducible Cre-recombinase mediated lineage tracing, it is clear that in the mouse retina there are at least two types of progenitors defined by the types of neurons they can generate: Ascl1+ and Ascl1- progenitors. Those progenitors that express Ascl1 do not produce retinal ganglion cells (RGC), though all other types of retinal neurons and the Muller glia are represented in their progeny. There also appears to be a further subdivision of the progenitor population based on Neurog2 expression: progenitor cells that express Neurog2 appear to be in their final mitotic cycle. The Ascl1 and Neurog2 lineage analysis leads to a more refined model of retinal progenitors (Figure 4). Although the expression of these bHLH transcription factors defines unique populations of retinal progenitors, it is not clear whether they are directly involved in specification. Ascl1, for example, has a more general role in driving the Notch-delta lateral inhibition system to maintain the progenitor pool (Nelson et al., 2009). Neurog2 has some role in early ganglion cell development (Hufnagel et al., 2010), but deletion of this gene does not produce a significant disruption in retinal development overall. Nevertheless, other bHLH genes, expressed in cells shortly after they exit the mitotic cycle, are critical for cell fate specification. Atoh7 (Math5) is transiently expressed in newly postmitotic cells, and deletion of this gene leads to a loss in ganglion cell differentiation (Brown et al., 2001); the transcription factors Ptf1a, NeuroD1 and Math3 are expressed in newly generated amacrine cells, and loss of one or more of these genes significantly reduces the number of amacrine cells in the retina (Fujitani et al., 2006; Inoue et al., 2002; Nakhai et al., 2007).

In addition to the bHLH genes, several additional transcription factors expressed in either progenitors or newly produced neurons are critical for the production of cell diversity. For example, Pax6, described above in the context of its role in the eye field and optic cup, is also important for the



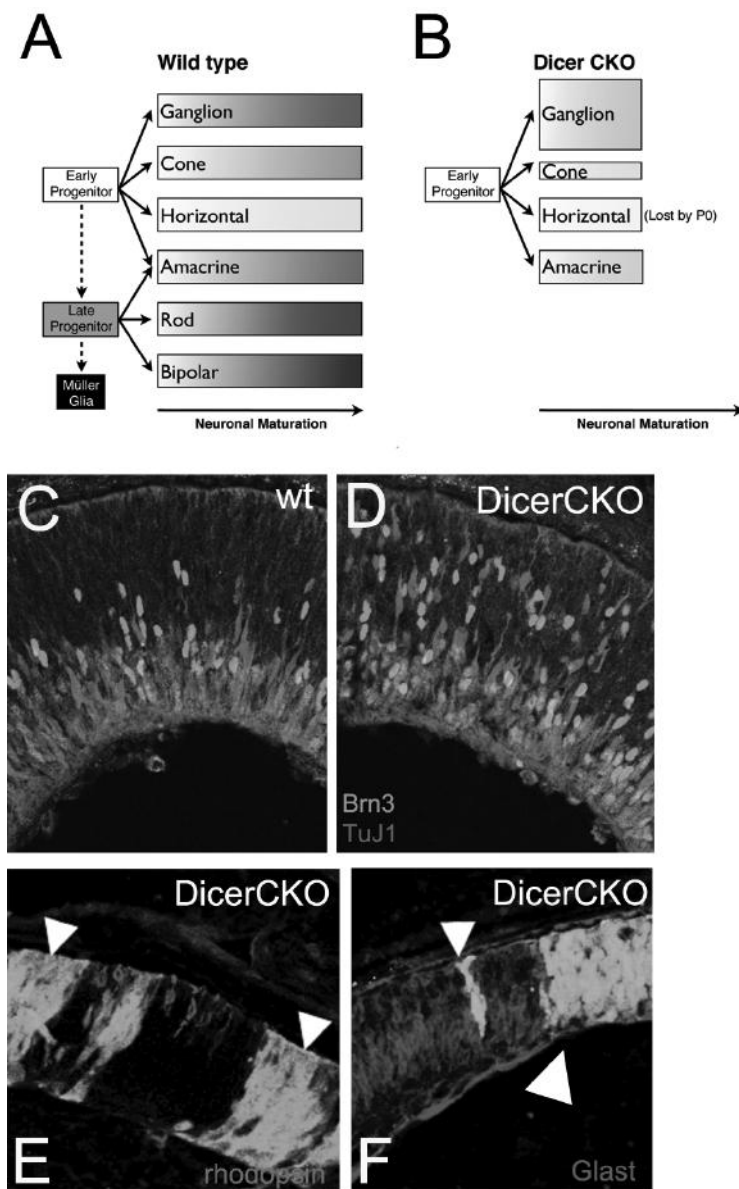
**Figure 4. Lineage of *Ascl1*<sup>+</sup> progenitors.** *Ascl1*<sup>+</sup> progenitors (diagram; green lineage) generate all retinal cell types except ganglion cells (RGC). By contrast, *Neurog2*<sup>+</sup> progenitors can give rise to all types of retinal neurons, including ganglion cells, but the clones are typically made of two cells suggesting that this transcription factor is expressed only in the last cell division whereas *Ascl1* is expressed when the cells can still divide multiple times. An example of an *Ascl1*<sup>+</sup> progenitor clone is shown to the right, where multiple cells (rods=r; bipolar cells=b; and amacrine cells=a) are shown (taken from (Brzezinski et al., 2011)).

development of specific types of retinal neurons. *Pax6* is expressed in most, if not all, of the multipotent retinal progenitors, and in postmitotic amacrine cells and horizontal cells. Targeted deletion of *Pax6* from retinal progenitors in mice after the stage of optic cup formation leads to the loss of all retinal cell types except amacrine cells (Marquardt et al., 2001). Conversely, *FoxN4* is a transcription factor that is expressed in only a subset of retinal progenitors, but deletion of this gene in mice causes the retinal progenitors to generate all retinal cell types except amacrine cells (Li et al., 2004). Thus, the combination of *FoxN4* and *Pax6* are necessary for the competence of progenitors to generate all retinal cell fates. However, these are not the only important factors; *Otx2* is expressed in photoreceptors and bipolar cells; targeted deletion of *Otx2* in mouse retinal progenitors leads to a retina with an overabundance of amacrine cells and no rods, cones or bipolar cells (Koike et al., 2007; Nishida et al., 2003). By contrast, deletion of another transcription factor that is expressed transiently in photoreceptors, *Blimp1/Prdm1*, leads to a retina with an overproduction of bipolar cells and very few rods and cones (Brzezinski et al., 2010). A loss of function mutation in *Chx10/Vsx2*, a gene that produces a paired-homeodomain transcription factor, eliminates

nearly all bipolar cells (Burmeister et al., 1996) while knockout of the bZIP transcription factor, *Nrl*, causes the developing rod photoreceptors to develop into short-wavelength sensitive cone photoreceptors (Mears et al., 2001). The cone photoreceptors in mammals can be further subdivided into S-cones and M-cones. The transcription factors thyroid hormone receptor- $\beta 2$  (TR $\beta 2$ ) and RXR- $\gamma$  promote M-opsin expression while inhibiting the expression of S-opsin (Ng et al., 2001; Roberts et al., 2005; Roberts et al., 2006). Deletion of TR $\beta 2$  leads to mice with no M-opsin cones, but only S-opsin cones. The way in which these transcription factors progressively subdivide the retinal progenitor progeny pie into finer slices is schematized in Figure 4C. There appears to be a hierarchy in the restriction process, with the first distinction being whether the newly postmitotic cell expresses Pax6 or Otx2. If the former, it can develop as an amacrine cell, a ganglion cell, a horizontal cell or a Muller glia. If the latter, it can develop as a rod or cone photoreceptor or as a bipolar cell. In the Otx2 population, expression of Prdm1 along with Otx2, specifies the cell to a photoreceptor fate, while the Otx2+/Prdm1- cells will develop as bipolar cells. The Pax6+/Otx2- "lineage" is not as clear at this time, but several of the key factors in the process have been placed on the diagram for their required roles in fate specification/differentiation of the respective cell types.

We have recently discovered a role for miRNAs in the progression of retinal progenitors from their "early" to "late" states. We were motivated in part by the classic work on miRNAs in *C. elegans*, in which these molecules were found to play a critical role in the regulation of a similar early-to-late transition in the generation of cell fates in the stem cell lineages. To test for a role of miRNAs in retinal development, we generated a retinal-specific deletion of *Dicer*, one of the critical enzymes in miRNA processing. For these experiments we used a line of mice with Cre-recombinase expressed specifically in a domain of the retina encompassing the peripheral third, starting at E11.5-12 ( $\alpha$ -Pax6-Cre). In addition to the floxed alleles of *Dicer* and the Cre, the mice also had a reporter for recombination so that we could follow the cells that had *Dicer* deleted. We further verified that *Dicer* was deleted in the retinal progenitor cells by measuring the levels of miRNAs in the retinas of these mice (Georgi and Reh, 2010; Georgi and Reh, 2011). We found a striking phenotype in the domains of retina that no longer had miRNAs: the progenitor cells did not express late progenitor markers, even into postnatal ages, and they continued to generate early cell types, like ganglion cells at a greater rate and long past their normal competence period for early cell fates. Those cell types normally generated later in development, like rods, bipolar cells and Muller glia were not generated even in postnatal day 6 mice, at the normal end of retinogenesis. By every measure, the progenitor cells without *dicer* appear to be stuck in an "early" progenitor state (Figure 5).





**Figure 5. MicroRNAs control the timing of retinal development.** Sequential production of retinal cell types and effects of miRNA deletions are shown (**A** vs. **B**). Conditional knockout of Dicer led to retinal ganglion cells being produced in excess (**C** vs. **D**) and rod photoreceptors (**E**) and Muller glia (**F**) being depleted in the affected regions (green). Markers used and colors are indicated in each panel (A and B modified from Georgi and Reh, 2011).

*Color image of this figure appears in the color plate section at the end of the book.*

## **Signals and the Regulation of Cell Production**

Notch is one of the key signaling pathways in developmental biology. As noted above, this signaling pathway plays critical roles at many stages of fly eye development. Many lines of evidence have shown key roles for Notch signaling in the vertebrate retina as well. Two homologs of *Drosophila* Notch, Notch1 and Notch3, are expressed by retinal progenitors throughout neurogenesis. The primary function of this pathway is to maintain the progenitor pool by preventing them from differentiating prematurely. The basic circuitry by which the Notch pathway prevents differentiation is as follows: Notch activates the expression of Hairy-Enhancer of split- (*Hes*)5 and *Hes1* via CSL/SuH/Rbpjk (Nelson et al., 2006) in retinal progenitors. The *Hes* transcription factors repress proneural bHLH genes (e.g., *Ascl-1* and *Neurog2*) (Hojo et al., 2000; Nelson et al., 2006) and this in turn prevents the cells from activating downstream elements of this neural differentiation cascade. Blocking the Notch pathway in retinal progenitors, using antisense oligonucleotides, conditional knockouts or small molecule inhibitors of gamma-secretase, which cleave Notch to generate its active form, leads to the differentiation of retinal progenitors into neurons of various types.

As noted above, the various types of neurons in the vertebrate retina are generated in a highly stereotypic sequence during histogenesis. Experiments in fly using temperature sensitive Notch mutants to inhibit Notch at specific times in development led to defects in differentiation of the cell types that are normally generated at the time of the temperature shift. Recently, it has been possible to inhibit Notch in a similar temporal manner in the vertebrate retina, and the results are remarkably similar. Taking advantage of a small-molecule inhibitor of the Notch pathway Nelson et al. blocked Notch at specific times in chick and mouse retinal development (Nelson et al., 2007). They found that inhibition of the pathway early in development led to an increase in the number of early generated cells, while inhibition of the pathway later in development led to a relative increase in those cells normally generated late in development. Thus, the timing of inhibition drives the formation of cells that are stage appropriate (Nelson et al., 2007).

There are additional similarities between Notch signaling in the fly and vertebrate eye. In *Drosophila*, the ligand for Notch, *Delta*, is expressed in the delaminating neuroblast in the proneural cluster; however, in the eye imaginal disc, *Delta* is expressed in a broad band of cells adjacent to the morphogenic furrow. This expression pattern has led to the model that instead of "lateral inhibition" the cells in the eye imaginal disc control differentiation through a "mutual inhibition." Recent analysis of *Dll1*, 3 and 4 expression in developing mouse retina shows that the primary ligand, *Dll1*, is not expressed by the differentiating neurons, but rather *Dll1* is expressed

by the mitotically active progenitor cells, the same cells that express Notch. So a patterning mechanism that works through mutual inhibition appears to function in the vertebrate as well (Nelson and Reh, 2008). This is likely the case throughout the CNS of birds and mammals. However, a complication with the simple view of Notch is that there are three ligands and two Notch receptors expressed in the developing retina; although these may convey similar signals cumulatively, there is some evidence that there may be cell-type specific information conveyed by different ligand-receptor pairs. In the spinal cord, Dll4 expressed in the progenitors of a specific subset of interneurons signals to neighboring progenitors to keep them from adopting their fate (Del Barrio et al., 2007). A similar situation might occur in the retina, given that Dll3/4 are expressed in subsets of progenitor cells (Nelson and Reh, 2008).

Further evidence for an instructive signal from Notch, rather than a passive signal comes from conditional deletion experiments of Notch1. When Notch is conditionally deleted from developing retina, the progenitors differentiate prematurely but, unexpectedly, there is a significant over-representation of photoreceptors in their differentiated progeny (Jadhav et al., 2006; Yaron et al., 2006). One interpretation of this result is that the Notch pathway is in some way instructive for directing progenitors to develop as cells other than photoreceptors. However, one caveat is that most of the progeny of retinal cell divisions are normally rod photoreceptors and so there is always a large bias in the progenitors for adopting this fate. Moreover, a photoreceptor bias was not observed in earlier experiments in which Notch was blocked by antisense oligonucleotides in embryonic chick retina (Austin et al., 1995) or when Notch was blocked in either embryonic chick or mouse with the gamma-secretase inhibitor DAPT (Nelson et al., 2007). This discrepancy may have to do with the fact that there are two Notch receptors in the retinal progenitors and in the conditional mutants only Notch1 was lost while in the DAPT experiments the overall Notch activity was inhibited. This explanation fits nicely with the idea expressed above that different Notch/ligand pairs may selectively control differentiation of specific cell types but further experiments are needed to test this hypothesis.

Another role for Notch in the vertebrate retina is in the generation of glia. The Muller glial cell is derived from the same progenitor population that gives rise to all the other neurons in the retina, but is in some ways "at the end of the line" in the process of histogenesis. As noted above, high levels of Notch signaling repress proneural transcription factors and, as such, should bias the cells towards a glial fate. Deletion of proneural/neural differentiation transcription factors, particularly in combination, leads to an over-production of Muller glia, as does over-expression of Notch intracellular domain or the Notch pathway effector Hes5 (Furukawa et al.,

2000; Hojo et al., 2000). In addition, loss of Notch function (or Hes5; Hojo et al., 2000) relatively late in retinal histogenesis leads to a reduction in the number of Muller glia. However, recent evidence has suggested that the function of Notch signaling in glial development is not simply a permissive one but instead activates glial-specific targets (Wang and Barres, 2000), particularly through non-canonical Notch signaling. It remains to be seen whether similar factors are involved in Muller glial differentiation.

Retinal progenitors also require signaling factors to regulate their proliferation and production of specific retinal cell fates (Levine et al., 2000). Retinal progenitor cells isolated from late stages of embryonic development, or from neonatal retina, respond to Egf, TGF- $\alpha$ , Fgfs, Shh, and several other mitogenic factors by increasing their proliferation (Anchan and Reh, 1995; Anchan et al., 1991; Lillien and Cepko, 1992). The proliferation of progenitors can be inhibited by treatments that raise cAMP (Taylor and Reh, 1990) and Tgf- $\beta$  signaling (Close et al., 2005). Several studies have also used *in vitro* techniques to identify factors that direct the progenitors to specific fates (Altshuler and Cepko, 1992; Davis et al., 2000; Kelley et al., 1994, 1995; Reh, 1992). Many of these studies have concentrated on rod photoreceptor differentiation. Since rod photoreceptors do not differentiate very well in low-density cell cultures, but do so in high density cultures or retinal explants, investigators have developed assays to screen for rod photoreceptor differentiation factors. Retinoic acid, taurine, Activin and Shh have all been shown to increase rod photoreceptor differentiation in some assays and in some species. Several of these have more recently been used to promote photoreceptor differentiation in embryonic stem cells after they have been directed to a retinal fate (see below).

## **Adult Vertebrates: The Ciliary Marginal Zone (CMZ)**

The development of the eye is a lifelong process in amphibians, fish and to some extent birds. In these animals, the retina continues to grow by the addition of new neurons of all types at the peripheral edge. Teleost fish can add up to one hundred fold more cells from the retinal margin than in the whole eye at hatching. The new retinal neurons are produced from a ring of cells at its peripheral edge, at the junction with the ciliary epithelium. The region of the eye that contains these cells is called the ciliary marginal zone (or CMZ) (Hollyfield, 1971). The cells in the CMZ of fish and frogs express a similar pattern of genes as do the early progenitor cells of the eye (see below). Most of the retina of the mature fish and frog (Reh and Constantine-Paton, 1983) is produced by the CMZ cells. Lineage tracing studies of these cells, similar to those done in embryos (Wetts and Fraser, 1988) demonstrated that they could produce clones of all types of retinal neurons, like the cells

in the early embryonic retina. These results, and others, suggest that the CMZ of fish and frogs contains true retinal stem cells.

CMZ cells in non-mammalian vertebrates express the EFTFs (Fischer and Reh, 2000; Perron et al., 1998), as well as the bHLH transcription factors, *Neurog2* and *Ascl1* (Perron et al., 1998). In addition, some of the CMZ cells respond to the mitogenic growth factors that stimulate embryonic progenitors to proliferate (Fischer and Reh, 2000; Moshiri et al., 2004). In fish, larval anuran and urodele amphibians the CMZ is highly productive; however, in birds it is greatly reduced (Fischer and Reh, 2000). In opossum, Kubota et al. (Kubota et al., 2002) reported a small cluster of BrdU incorporating cells at the junction between the ciliary epithelium and the retina, even in animals one month of age (Kubota et al., 2002); however, in the eutherian mammalian retinas that have been analyzed there are few, if any, mitotically active cells in this location. It is not known whether the proportion of cells in the fish or frog CMZ represent true retinal stem cells with the capacity to proliferate throughout the animal's lifetime and what proportion of them are progenitors with a more limited proliferative potential. Most of the bird retina is generated during embryonic development and relatively few neurons are generated by the CMZ after hatching. Although there have been no studies of aged birds, new cells are generated at the peripheral edge of the retina in chickens up to one month posthatch (Fischer and Reh, 2000) and in the quail retina for up to one year (Kubota et al., 2002).

Although the CMZ is greatly reduced or absent in the mammalian eye, some studies suggest that this zone may be repressed *in vivo* and can be activated by an appropriate mitotic stimulation. Mice with a single allele of the *Patched* (*Ptch*) gene, a negative regulator of *Shh* signaling (Moshiri et al., 2004), results in a small number of proliferating cells at the retinal margin even during adulthood. Crossing *Ptch*<sup>+/-</sup> mice with mice that have an inherited photoreceptor degeneration stimulates proliferation in this region reminiscent of the proliferative response of the CMZ cells of lower vertebrates to retinal damage. There is also evidence that, in some cases, injection of mitogens into the rat eye can stimulate BrdU uptake of cells in this region even in normal animals though the labeled cells have not been shown to differentiate into neurons as they do in non-mammalian vertebrates (Close et al., 2005; Close et al., 2006; Zhao et al., 2005).

## RETINAL REGENERATION

Among the most dramatic examples of organ regeneration in vertebrates occurs in the eyes of newts. Newts and salamanders are capable of regeneration of many of their tissues, including parts of the eye. If the neural retina is experimentally removed in newts, it is restored structurally

and functionally within five weeks. Retinal regeneration in amphibians occurs through the phenomenon of transdifferentiation of the pigmented epithelium into neural retinal progenitors (Okada, 1980; Stone, 1950). Soon after retinal removal, the pigmented epithelial cells re-enter the mitotic cell cycle. The cells lose their pigmentation, and instead express genes normally present in retinal progenitors, like the EFTFs and proneural bHLH transcription factors. The pigment epithelial derived progenitors produce new retinal neurons in the same pattern as present in normal retinal histogenesis (Reh et al., 1987; Sakaguchi et al., 1997), recapitulating the normal process of development. Ultimately the newly regenerated retinal ganglion cells re-grow connections with the optic tectum to restore visual behavior.

The basic cellular transformations that occur during retinal regeneration in amphibians are well-characterized. In addition, recent studies have explored the molecular mechanisms underlying this phenomenon. As noted above, as the pigment cells proliferate following retinal removal or damage, they express many genes normally present in retinal progenitor cells, but not normally expressed by the pigmented epithelial cells (Reh et al., 1987; Sakami et al., 2005). Indeed, it is likely that the RPE-derived progenitors de-differentiate to a state like that of the cells in the early optic cup, since they can produce all the various cell types normally present in the mature retina and can do this multiple times. It is possible that these cells go through a stage where they resemble stem or “founder” cells because the RPE cells can regenerate the entire retina in some species, up to four complete times (Stone and Steinitz, 1957). A similar process of de-differentiation of the pigmented epithelial cells occurs in embryonic chick and mammals also leading to retinal regeneration. However, the ability of RPE cells to transdifferentiate into retinal stem or progenitor cells is present only in the early stages of eye development (Park and Hollenberg, 1993; Pittack et al., 1997; Zhao et al., 2002). A key stimulus for retinal regeneration from the RPE in both amphibians and chick embryos is Fgf (Park and Hollenberg, 1989; Pittack et al., 1991; Sakaguchi et al., 1997) and at least in the embryonic chick, Shh also plays a role in this process (Spence et al., 2004).

The ciliary body is another region of the eye that has been proposed to contain retinal stem cells or progenitors. The ciliary body is derived from both the neural tube (the inner epithelial layers) and the neural crest (the muscle and connective tissue layers). The two-layered ciliary epithelium is an anterior extension of the neural retina and RPE. Several studies have tested whether the ciliary epithelium contains neurogenic cells, or cells that could be induced to produce neurons. In posthatch chicks, the cells of the ciliary epithelium in the chick express the EFTFs, Chx10 and Pax6, like the CMZ cells. Intraocular injections of insulin, Fgf2 and Egf in the chick stimulate the proliferation of cells in the ciliary epithelium and

some of these dividing cells differentiated into neurons with long axons (Fischer and Reh, 2003). The neurons that develop in this region following growth factor treatments resemble amacrine cells and ganglion cells; no photoreceptors were observed. These results show that at least in birds, the ciliary epithelium contains neurogenic cells.

Some studies have also suggested that the mammalian ciliary epithelium has some ability to generate neurons. Although the ciliary epithelium of mice and monkeys has few actively proliferating cells, several groups (Ahmad et al., 2000; Fischer and Reh, 2001; Inoue et al., 2010; Tropepe et al., 2000) have found that pigmented cells from the ciliary epithelium or iris can form neurospheres that express some neuronal markers when dissociated and cultured for extended periods. The sphere-forming potential of these cells has led to the claim that they are retinal stem cells, analogous to the sphere forming neural stem cells isolated from other regions of the CNS (Ahmad et al., 2000; Tropepe et al., 2000). In addition, the ciliary epithelium of human eyes also contains sphere-forming cells (Coles et al., 2004), which can be expanded *in vitro* and transplanted. More recent studies, however, have challenged the original claims that there are retinal stem cells in the ciliary epithelium of mammals. Even in the neural stem cell field, the ability to form spheres no longer appears to be a reliable assay to predict stem cell potential. Moreover, Dyer and colleagues demonstrated that the sphere-forming cells from the mouse ciliary epithelium retain their ciliary epithelial identity, only very low levels of neuronal genes are expressed and the morphology of the progeny of these ciliary epithelial derived sphere-forming cells does not resemble that of retinal neurons (Cicero et al., 2009). Thus, while ciliary epithelial cells can be cultured as spheres, these do not appear to resemble the retinal stem cells of non-mammalian vertebrates or even the retinal progenitors of embryonic retina.

In addition to the sources of regeneration described above, another potential source for regeneration of the retina after injury is a glial cell known as Müller glia. The Müller cells are the primary glia of the retina; they are generated by the same multipotent retinal progenitors that produce the retinal neurons. Fish have the ability to regenerate all retinal cell types after damage; the source of the new neurons after damage is the Müller glia (Hitchcock and Raymond, 1992). When the retina is damaged in fish, the Müller glia undergo a robust proliferation and up-regulate aspects of the retinal progenitor gene expression program (Bernardos et al., 2007; Fausett and Goldman, 2006; Fimbel et al., 2007). One of these progenitor genes, the proneural bHLH gene *Ascl1a* is normally not expressed by Müller glia but after damage its expression increases dramatically. *Ascl1* is required in the Müller glia to regenerate neurons: if it is knocked down in the glia after retinal injury, retinal regeneration fails (Fausett et al., 2008; Ramachandran et al., 2010). Other neural progenitor genes, such as *Notch* and *Pax6*, are also

increased in the Müller glia after damage (Qin et al., 2009; Raymond et al., 2006; Thummel et al., 2008; Yurco and Cameron, 2007). Several signaling factors are important for retinal regeneration in fish; most recently, HB-Egf has been shown to be required for retinal regeneration in fish, but midkine- $\alpha$  and - $\beta$  and ciliary neurotrophic factor (CNTF) are also critical (Calinescu et al., 2009; Kassen et al., 2009).

Although the fish retina has been known to regenerate since over 50 years, more recently Müller glial cells of posthatch chicks were found to also reenter the cell cycle after neurotoxic damage (Fischer and Reh, 2001, 2002). After retinal damage, Müller glia proliferate in chick much like they do in fish after damage, and some of the proliferating Müller cells express progenitor genes, including Chx10, Pax6, Cash1/Ascl1, FoxN4, Notch1, Dll1, and Hes5 (Fischer and Reh, 2001; Hayes et al., 2007). Thus, the Müller glial cells of chicks can enter a progenitor state much like that of fish after retinal injury. Although the initial stages are similar, there are some differences. The Müller glial-derived progenitors of fish undergo several rounds of cell division after damage; by contrast, in the chick, most of the Müller glia divide only once following injury. However, while in fish the majority of Müller glial cells express the progenitor state after injury, in the bird retina only a subset of them do so. Moreover, an even smaller subset of these progenitor-like cells produce neuronal cells, expressing markers of amacrine cells, bipolar cells and ganglion cells (Brn3 and neurofilament). Most of the Müller glial progeny remain as progenitor-like cells, but do not progress to neuronal differentiation.

What about mammals? Do Müller glia show any signs of regenerative ability? Mammalian Müller glia respond to injury by changes in their pattern of gene expression that have been collectively called “reactive”; however, in most mammalian species that have been examined, only a small percentage of the Müller glia re-enter the mitotic cycle (Dyer and Cepko, 2000). Nevertheless, Müller glia can be isolated and grown *in vitro* after light damage in rats (Sarthy, 1985) and can proliferate *in vivo* in cats following retinal detachment (Lewis et al., 2010). Studies have reported that a small number of Müller glia will re-enter the mitotic cycle after retinal damage (Ooto et al., 2004; Wan et al., 2008), and Müller glial proliferation is stimulated by following the damage with mitogen treatment (Close et al., 2006; Karl et al., 2008; Osakada et al., 2007; Takeda et al., 2008). Another difference between the mammalian glial response to injury and that of non-mammalian vertebrates is that in fish (and to some extent birds) Müller glia de-differentiate to produce progenitors with a developmental pattern of gene expression (see above). Karl et al. (Karl et al., 2008) analyzed a panel of genes by RT-PCR in mouse retinas after injury to determine whether they undergo a similar de-differentiation process. Although Pax6, Notch pathway genes, Sox2 and nestin are up-regulated in the Müller glia after



NMDA damage in mammals, many other progenitor genes are not (Das et al., 2006; Karl et al., 2008; Osakada et al., 2007; Wan et al., 2008). Do the Müller glia regenerate new neurons in the mammalian retina? (Ooto et al., 2004) 2–3 weeks after retinal injury in adult rats, some BrdU+ cells were reported to express markers of bipolar cells and photoreceptors (Ooto et al., 2004) and a combination of NMDA and mitogens stimulated Müller glial proliferation and differentiation of their progeny into BrdU+ cells with markers of amacrine cells such as Calretinin, NeuN, Pax6, Prox1 and GAD67-GFP (Karl et al., 2008). In these studies it was possible to verify that the BrdU+ cells were amacrine cells by the use of mice that expresses GFP in amacrine cells (GAD67-GFP). Other types of damage have been used to study the potential for mammalian retinal regeneration including Wnt3a (Osakada et al., 2007), MNU damage (Wan et al., 2008; Wan et al., 2007), and alpha-amino adipic acid (Takeda et al., 2008). These studies have shown some evidence for regeneration, though it appears to be quite limited.

### **Embryonic Stem Cells and Induced Pluripotent Stem Cells to Generate Retinal Neurons**

Although the retinas of non-mammalian vertebrates can regenerate very well, particularly in amphibia and fish, this potential is almost non-existent in the mammal. Therefore, attempts to repair damaged retina in mammals has focused on transplantation of fetal retinal tissue (MacLaren et al., 2006; Radtke et al., 2004). However, it is difficult to use primary human fetal retinal tissue as a therapy and as a result several investigators are developing methods to direct human embryonic stem cells (hESCs) (Lamba et al., 2008) and more recently human induced pluripotent stem cells (iPSCs) to retinal progenitors (Bull and Martin, 2011). Embryonic stem cells are derived from the inner cell mass of the blastocyst. These cells can self-renew indefinitely (Thomson et al., 1998) and in theory can differentiate into all cell types in the body. Induced pluripotent cells (iPSCs) have become a popular alternative to ESCs, since they are now easily generated from fibroblasts with a combination of reprogramming factors and behave similarly to hESCs.

There are now several protocols for directing mouse ESCs towards retinal differentiation (Aoki et al., 2006; Hirano et al., 2003; Ikeda et al., 2005; Meyer et al., 2004; Sugie et al., 2005; Tabata et al., 2004; Zhao et al., 2002). Early studies using retinoic acid for neural induction (Fraichard et al., 1995) or basic fibroblast growth factor (FGF-2) and a combination of insulin, transferrin, selenium and fibronectin (ITSFn) yielded a high proportion of neuroepithelial cells, which can produce neurons and glia. Placing the neurally-induced ES cells in a neurogenic micro-environment (Sugie et al., 2005; Zhao et al., 2002) or in degenerating retina (Meyer et

al., 2004) promoted further retinal differentiation. Neurally-induced ES cells, transplanted to a degenerating retina, allowed cells to penetrate the retina and acquire neuronal morphology. However, the transplanted cells did not express photoreceptor markers (Meyer et al., 2004). When ES-derived embryoid bodies are cultured with the stromal cell line PA6 they differentiate into retina and RPE (Aoki et al., 2006; Hirano et al., 2003). Retinal differentiation of ESCs can also be promoted by over-expressing the eye field transcription factor Rx (Tabata et al., 2004). A combination of three factors, lefty-A, Dkk1 and Activin A, was used to direct mouse ESCs to a retinal identity (Ikeda et al., 2005), causing nearly 30% of all cells to express Pax6, Rx and two key EFTFs (see above) with some cells also expressing the markers of photoreceptors rhodopsin and recoverin. Most recently, a protocol has been developed that uses very limited conditions to produce highly organized retina from mouse ESCs (Eiraku et al., 2011). In this protocol, antagonizing BMP signaling biases embryoid bodies to neural fates (and by “default” anterior fates) and the complex neuroepithelium presumably provides the other patterning signals to direct part of the epithelium to form optic vesicles and optic cups that can be isolated by microdissection. These results highlight the fact that we still do not understand all the complex patterning signals present in the developing brain and how these impact eye development.

The manipulation of key developmental signaling pathways was also used in the design of a protocol for generating retinal cells from human ES cells. Human ESCs were efficiently directed to a retinal identity using a combination of a Bmp inhibitor (Noggin), a Wnt inhibitor (Dkk1), and insulin-like growth factor (Igf-1) (Lamba et al., 2006). The retinal cells produced with this protocol expressed key eye field transcription factors and several early markers of photoreceptors, e.g., Crx, Nrl, and recoverin. A similar approach was used with alternate antagonists of Wnt and Bmp/nodal pathways (Osakada et al., 2008). The similarity of these protocols for human ESCs and mouse ESCs indicate that eye field induction in human and mouse ES cells is quite similar as antagonists to Wnt and Bmp are normally important to produce anterior neural tissue (see above). It is also important to note that markers of cerebral cortex, like Emx1, are not induced by these protocols. Additional evidence for their specificity comes from a recent microarray study in which the ESC-derived retinal cells were directly compared with fetal human retinal cells (Lamba and Reh, 2011) showing to be highly similar in their pattern of gene expression although other tissues derived from the optic cup, the ciliary epithelium and RPE were also produced by the Lamba protocol. Both the Lamba and Osakada protocols reported that early stages of photoreceptor development occur in the hESC-derived retinal cultures. More recently, combinations of small molecules that modulate the same developmental Wnt and Bmp pathways have been

used in retinal differentiation (Osakada et al., 2009). Another protocol for directing hESCs and iPSCs (Meyer et al., 2009) relies on the identification and selection of the eye field cells by their characteristic spheres. These investigators found that the cells follow the same developmental stages as those that occur in eye development *in vivo* (Meyer et al., 2011; Meyer et al., 2009). One assay used to assess directed differentiation is the ability of the differentiated cells to integrate into host retina following transplantation. The ability of cells to integrate following transplantation is a key proof of principle for cell-based therapies. The first group was that of Reubinoff to show that ES-derived retinal cells can be successfully transplanted (Banin et al., 2006). However, their protocol to direct ESCs to a retinal fate was not very efficient, so it was difficult to determine whether retina-specific cell types were differentiating in the transplanted cells. Since that time, human ESC-derived photoreceptors have been successfully transplanted into wild type and congenitally blind mice, with good integration and even some functional restoration (Lamba et al., 2009a; Lamba et al., 2009b).

Human iPS cells circumvent some of the ethical issues of ES cells in that destruction of embryos is not necessary for their derivation. The fact that iPS cells can be generated from adult human fibroblasts allows investigators to generate pluripotent cells from patients with specific inherited disorders; potentially, specific retinal disease models can be generated *in vitro*. Several investigators have demonstrated that iPS cells can be directed to differentiate along the retinal lineage using protocols similar to those developed for ES cells (Hirami et al., 2009; Lamba et al., 2010; Meyer et al., 2011; Meyer et al., 2009). The iPSC-derived retinal cells develop many characteristics of cells derived from fetal retina. In addition, when transplanted to normal mouse retinas, the iPSC-derived retinal cells can incorporate into the host retina (Lamba et al., 2010). The efficiency of retinal differentiation varies among iPSC lines. In the best cases, iPSCs are equal or better than many ES lines for the generation of retinal cells.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Our understanding of the key molecular events in eye and retinal development has advanced at a rapid rate over the last 20 years. However, there are many critical gaps and inconsistencies in the current models and more systematic, integrative approaches will be important for the field to move to the next level. We now know many of the transcription factors that control cell identities in the retina and also have identified some of the key signaling molecules that are involved, but little is known about the connections between signals and transcription factors. In some cases, the investigations of ES cells can inform developmental biology, as evidenced by the fact that optic cups can form in mouse ES cells, presumably

without extraocular mesenchyme cells, whereas these have been shown to be critical for appropriate patterning of the ocular epithelium in chick embryo experiments. The current trends in regenerative medicine and reprogramming should also help provide a better understanding of the limits to regeneration in the mammalian retina and possibly even methods for reinvigorating the regenerative program in mammals.

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# Progenitor Cells in Embryonic and Adult Lungs

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## SUMMARY

The lung is a complex organ that requires interactions between tissues and cells of various lineages to efficiently perform the task of gas exchange. Here we review the general development of the lung, focusing on what is known about the embryonic progenitor lineages that give rise to the mature cell types found in the adult lungs. While the epithelial lineages have been well characterized in terms of the major contributing cell populations, much less is understood about the mesenchymal development, including the lineages that give rise to the vascular, smooth muscle, and lung haematopoietic cells. Adult multipotent lineages and their role in maintenance, repair, and regeneration will also be examined. Characterization of these adult populations is important for understanding the potential of the various lung lineages for differentiation into functional cells that could be used to regenerate the lung.

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*List of abbreviations after the text.*

## INTRODUCTION

### Lung Development

The task of gas exchange that occurs in the lung requires a complex interplay of the epithelial, vascular, and mesenchymal cells. The airway lining cells are derived from the ventral foregut endoderm, which migrate up through the primitive streak during gastrulation; in the mouse this occurs by embryonic (E) day 7.5 (Wells and Melton, 1999). From this primitive gut tube, organs begin branching off, with the first lung specific marker (Nkx2.1) present at E9.5 in the mouse (5 weeks in human embryo) (Warburton et al., 2000). Models have been proposed for endodermal organ specification, whereby different thresholds of FGF coming from the developing cardiac mesoderm, pattern the foregut. Based on this model, no or minimal signaling results in a 'default' pancreatic fate and increasing concentrations specify liver, lung, and thyroid, respectively (Serls et al., 2005). Less is known about the timing of the mesodermal contributions to lung development, but reviews of multiple studies suggest that the mesoderm-derived progenitors of the pulmonary vasculature and pulmonary smooth muscle may be present within the developing lung as early as E10.5, with more specified vascular and smooth muscle cells arising closer to or after E11.5 and E13.5 (Morrisey and Hogan, 2010; Rawlins, 2011). Haematopoietic cells establish themselves in higher populations with more significant contributions during the postnatal stages of lung development and mostly in response to the continual cycle of air and its particulate contents.

Lung development itself can be divided into six stages. Initial specification occurs in the *embryonic stage* (3.5–7 weeks in humans, 9.5–14.2 days in mice). In humans, the endodermal bud elongates caudally then bifurcates into the left and right primary bronchi. After this occurs, the original diverticulum moves rostrally and separates the foregut into two tubes, the dorsally located esophagus and the more ventral trachea. At this point the two lung buds grow along the posterior-ventral axis into the surrounding splanchnic mesenchyme and begin to branch into the secondary bronchi (two buds on the left and three on the right). In the mouse this process occurs in a slightly different order. The initial diverticulum from the foregut is composed of two buds. Before elongation into the surrounding mesenchyme, the foregut tube invaginates and pinches off into two tubes, the trachea and esophagus. After this separation has occurred the primary bronchi grow further into the splanchnic mesenchyme with the right bud branching into four secondary bronchi and left remaining un-branched (Rutter and Post, 2008). After initial branching occurs the lung enters the *pseudoglandular stage* (5–17 weeks in humans, 14.2–16.6 days in mice). This stage is characterized by development of blood vessels and

continued development of the bronchial tree by dichotomous branching. Specific cells within each compartment begin differentiating at this point (Roth-Kleiner and Post, 2003). During the *canalicular stage* (16–26 weeks in humans, 16.6–17.4 days in mice), the formation of the conducting airway is complete and the respiratory epithelium begins to develop into the respiratory bronchioles and alveolar ducts. The capillary bed also increases at this stage and marks the beginning of the future blood-air interface. The *saccular stage* (24–38 weeks in humans, 17.5 days–5 days post natal in mice) is critical for survival after birth. The peripheral airspaces distal to the terminal bronchioles expand at the expense of the mesenchyme to allow for more efficient gas exchange. Preparation for alveolarization occurs, marked by the deposition of elastic fibers at the sites where secondary septa will form. Mature surfactant is produced by the type II alveolar epithelial cells. The *alveolar stage* occurs largely postnatally in the mouse and human (36 weeks–2 years postnatal in humans, 4–14 days postnatal in mice) and is characterized by the appearances of low ridges on both sides of the saccular walls, referred to as secondary septa. Further growth of these septa into the airspace subdivides the saccules into alveoli and serves to increase the surface area available for gas exchange (Roth-Kleiner and Post, 2003; Rutter and Post, 2008; Warburton et al., 2010). The final stage of lung development is *microvascular maturation* (birth–3 years in humans, 14–21 days postnatal in mice), where the relatively thick bilayered secondary septa are fused into a capillary monolayer with minimal interstitial tissue left in the walls to impede gas exchange.

Throughout the entire process of lung development, cells in each of the compartments (epithelial, mesenchymal, and vascular) become increasing lineage restricted and specialized according, primarily, to positional cues from the surrounding tissues. The immature cells that give rise to the terminally differentiated population are often referred to as embryonic progenitor cells, as these are a transient progenitor population. When reviewing material regarding ‘stem cells’ it is important to define what is being discussed as this general term has been used to describe everything from embryonic stem cells in an undifferentiated state, to progenitor cells within a specific organ that have the ability to differentiate into a limited number of cell types within that organ (see Glossary). The generally accepted convention is that ‘stem cell’ refers to a cell that has a capacity for unlimited self-renewal as well as the ability to make at least one other cell type (Watt and Hogan, 2000). In most organs, including the lung, an intermediate cell type exists with a more limited proliferative capacity and restricted differentiation ability. This is commonly referred to as a transit amplifying cell, adult stem cell or progenitor cell (Watt and Hogan, 2000). For the purposes of this review, the term ‘progenitor cell’ will be used. The following criteria have been suggested for defining a stem cell population

within the lung as: 1) slow cycling, 2) highly proliferative, and 3) multipotent for its niche (Borthwick et al., 2001b; Liu and Engelhardt, 2008a).

## EPITHELIAL PROGENITOR CELLS IN THE FETAL LUNG

### Embryonic Lung Formation

The lining of the airway is composed of a diverse population of epithelial cells whose structure and function is directly related to their location within the lung. As development occurs definitive endoderm cells become increasingly lineage restricted as a result of cues from surrounding cells, particularly the mesenchyme (Alescio and Cassini, 1962; Deimling et al., 2007). During the embryonic stage, the lung buds consist of an unpatterned epithelial lining (Rawlins, 2008). Progenitors in the lung can divide symmetrically to give rise to two identical progenitors or differentiated daughter cells or asymmetrically resulting in one progenitor and one differentiated daughter cell. The cues that direct these types of cell divisions ultimately control the cell composition, size and shape of the adult lung (Rawlins, 2008).

Most of what is known about progenitor proliferation and expansion comes from early developmental studies looking at mutant mice with specific gene knockouts. The forkhead/winged helix (Fox) family of transcription factors play a large role in endoderm development with some specifying distal lung. Conditional deletions of *Foxa1* and *Foxa2* have global epithelial defects, including smaller than expected lungs with decreased branching and differentiation (Wan et al., 2005). *Foxp2*<sup>-/-</sup> and *Foxp1*<sup>+/-</sup> mice also have a small lung with decreased proliferation and *Nmyc* expression (Shu et al., 2007). Given the role of *Nmyc* in maintaining a distal population of undifferentiated, proliferating progenitor cells this suggests that *Foxp1* and *Foxp2* have an upstream role in maintenance of the progenitor compartment (Okubo et al., 2005; Rawlins, 2008). Deletion of epithelial bone morphogenetic protein receptor 1a (*Bmpr1a*) or its ligand *Bmp4* results in small lungs with decreased *Nmyc* and *Foxa2* expression suggesting autocrine epithelial signaling is in part responsible for control of the distal epithelial progenitor population (Eblaghie et al., 2006).

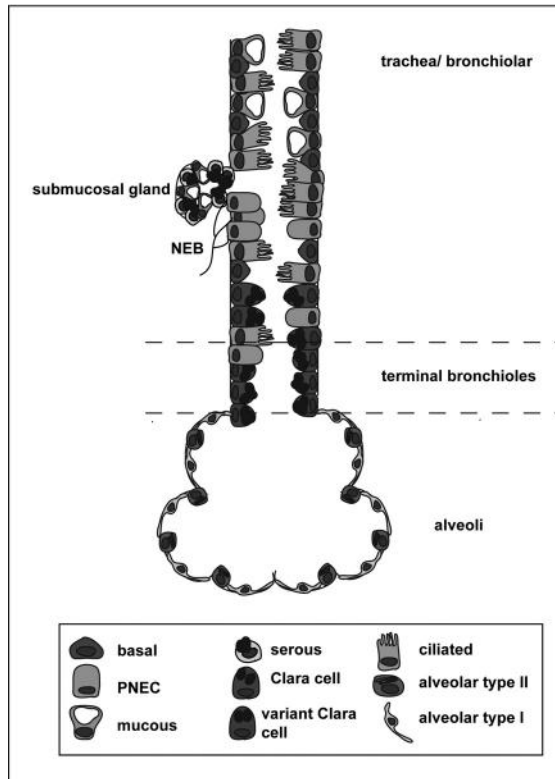
Sonic hedge hog (Shh) expression in the distal lung epithelium also plays a role in distal lung proliferation and branching morphogenesis and is another likely candidate for controlling the progenitor compartment in the lung (Pepicelli et al., 1998). Expression of non-canonical Wnt pathway member *Wnt5A* in the distal tips of the developing lung helps balance between proliferation and differentiation. Targeted deletion of *Wnt5a* results in expanded distal epithelial and mesenchymal compartment at the expense of functional, terminally differentiated cells (Li et al., 2002). The interactions between these signaling pathways in controlling the



progenitor cells within the epithelial compartment has yet to be determined, but it is likely a complex process involving common regulators as well as secondary signaling molecules that have yet to be identified (Rawlins, 2008). Other genes seem to have a more global role in differentiation of the lung epithelium. *Foxa1*<sup>-/-</sup>, *Foxa2*<sup>-/-</sup>, and *Nkx2.1*<sup>+/-</sup>, *Gata6*<sup>+/-</sup> mutants both have severe global lung defects in epithelial differentiation but their specific downstream targets have yet to be elucidated due to the ubiquity of their expression during early lung development (Wan et al., 2005; Zhang et al., 2007). Mesenchymal signaling is also important in proliferation, as *Fgf10* is a potent mitogen for the lung epithelium during branching morphogenesis. Hypomorphic mice for *Fgf10* have reduced epithelial cell proliferation during the pseudoglandular period of development (Ramasamy et al., 2007), while overexpression of *Fgf10* during lung development causes epithelial progenitors to arrest in an undifferentiated state (Nyeng et al., 2008). These results suggest a role for *Fgf10* in inhibiting differentiation and promoting self-renewal in epithelial progenitors and also highlight the importance of cross-talk between the epithelium and mesenchyme during lung development.

### **Proximal-Distal Patterning of the Airways**

In the mature lung, epithelial lineages are arranged in a proximal-distal fashion along the airways in a manner that reflects function (Figure 1). Earlier studies using developing rat lungs have shown that lineage restriction begins to occur in the epithelium as the lung progresses from the pseudoglandular to cannicular phases of lung development. Cells in the most proximal part of the lung lose the ability to respond to cues from the surrounding mesenchyme that are inducing cells to the distal lineage (Shannon, 1994; Shannon et al., 1998). It has been suggested that while this proximal-distal patterning occurs, the most distal epithelial cells are serving as the progenitor population for the entire lung. In this model, as the lung branches, descendants of the distal tip progenitors are left behind in the stalks where they begin to differentiate while the self-renewing progenitors remain in the tips and continue to divide (Rawlins, 2008). Evidence for this model comes from DNA labelling experiments using BrdU. This technique allows cells that are cycling slowly, or retaining the label, to be visualized after an appropriate washout period. Cells with a more rapid turnover lose the label more quickly so that after the washout only slow cycling cells remain labelled (Bickenbach, 1981). When the lung epithelial cells were labelled with BrdU under a short pulse, the distal cells were found to incorporate BrdU at a higher proportion than the rest of the lung, suggesting they have slower cell cycle kinetics (Okubo et al., 2005).



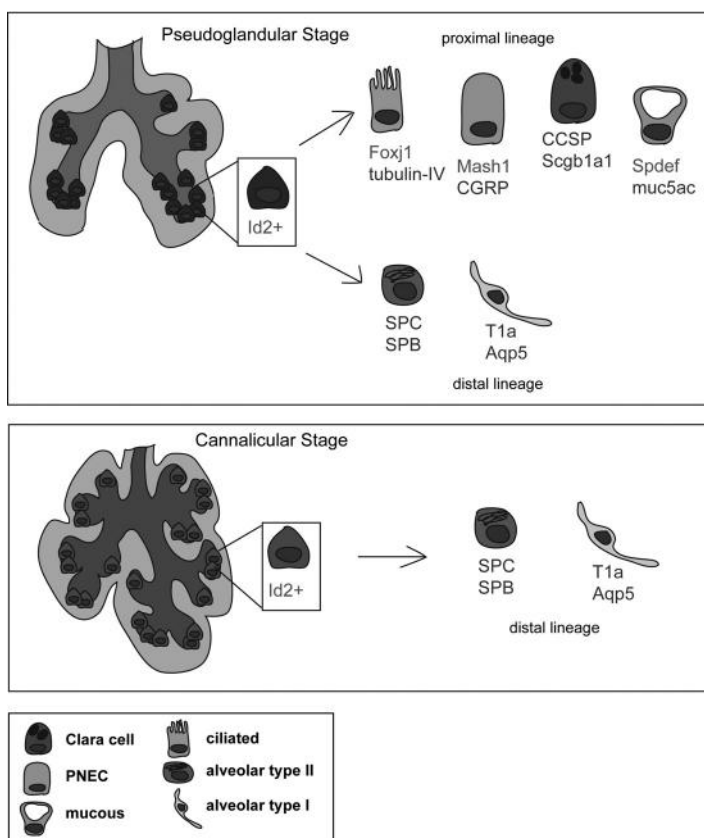
**Figure 1. Airway epithelial cells of the lung.** The tracheobronchial region of the conducting airways is lined with a pseudostratified columnar epithelium containing basal, ciliated, mucous, and occasional Clara cells. Submucosal glands lie in close proximity to neuroepithelial bodies (NEB) and serous and mucous cells. The bronchioles and terminal bronchioles are lined primarily with Clara cells, with occasional pulmonary neuroendocrine cells (PNEC), basal and ciliated cells. This area of the lung transits from a simple columnar to a low columnar/cuboidal epithelium. The alveoli are lined with very flat alveolar type I cells with a basal membrane fused to adjacent capillaries as well as cuboidal type II cells, which facilitates gas exchange. The conducting airway is depicted in blue and purple, while the gas exchange portion is shown in red and pink.

*Color image of this figure appears in the color plate section at the end of the book.*

This distal cell population has also been shown to have a unique pattern of gene expression including high levels of transcription factors *Nmyc*, *Id2*, and *Sox9*, and high levels of growth factors of the *Fgf*, *Wnt*, *Bmp*, and *Shh* signaling pathways, which are candidates for controlling the progenitor population (Bellusci et al., 1996; Liu and Hogan, 2002; Shu et al., 2005). *Id2* (inhibitor of differentiation 2) has been found to be expressed at high levels in the distal tip epithelial cells of the mouse prenatally. Knock-in experiments using an *Id2*-reporter lines revealed that early in the pseudoglandular phase

of development, Id2 cells contributed to the Clara, ciliated, neuroendocrine as well as the distal epithelial cells (Rawlins et al., 2009). Interestingly, when Id2+ cells were labelled later in the canalicular stage, these cells only contributed to the alveolar compartment suggesting that Id2+ cells represent an embryonic progenitor for the entire lung that becomes lineage restricted and then turns off at birth (Figure 2) (Rawlins et al., 2009).

*Wnt* signaling has been shown to play a role in regulating progenitor proliferation as well as proximal-distal patterning. Deleting  $\beta$ -catenin in the lung during the pseudoglandular stage of development results in a



**Figure 2. Multipotent Id2+ distal tip cells acts as an embryonic progenitor.** During the pseudoglandular phase of lung development, Id2+distal tip cells can contribute to the proximal and distal lineage (top). As the lung progresses into the canalicular phase, Id2+cells become lineage restricted and only contribute to cells found in the distal lung (bottom). The adult lung does not appear to retain this multipotent population, indicating the Id2+ cells only act as embryonic progenitors. The airway (blue) and surrounding mesenchyme (gray) are depicted.

*Color image of this figure appears in the color plate section at the end of the book.*

mature lung composed primarily of proximal cells (Mucenski et al., 2003). Similar results were obtained when *Wnt* signaling was blocked using *Dickkopf1* (Shu et al., 2005). In both cases, while proximal cells were made at the expense of distal cells, the overall proliferation of the lung remained unchanged indicating the role of the *Wnt* pathway in differentiation was independent of proliferation. Notch and Bmp signaling also appeared to play a role in cell-fate decisions. Overexpression of dominant negative Bmp receptors or the use of Bmp antagonists results in a loss of the distal phenotype (Lu et al., 2001; Weaver et al., 1999). Notch1 is highly expressed in the distal epithelium during the pseudoglandular phase of lung development (Post et al., 1996); however, misexpression of Notch later in development prevented the differentiation of the distal progenitor cells and resulted in the formation of cysts that lacked mature cells from the alveolar lineage (Guseh et al., 2009). Notch appears to be having an effect at least in part through *Sox2*. In lung development, *Sox2* is expressed in the non-branching regions of the epithelium and serves as a marker for cells that are committed to a proximal cell phenotype (Gontan et al., 2008; Ishii et al., 1998; Que et al., 2007). Pharmacological inhibition of Notch signaling in the early lung epithelium resulted in inhibition of the proximal cell fate with a reduction of *Sox2* expression domain in the developing airways (Tsao et al., 2008). These data suggest that there is a default of a distal cell fate for the lung and that antagonism of these default pathways occurs during proximal specification. This fits with the hypothesis of distal tip cells having progenitor capacity for the entire lung as the lung would attempt to retain this population until cues from the mesenchyme and vasculature signal that it is a developmentally appropriate time for differentiation.

As this process of branching and patterning occurs, differentiated cell types begin to appear along the airway with proximal cells exhibiting mature phenotypes prior to the distal lung. Early specification results in lineages from the tracheobronchial and bronchiolar lineages. The tracheobronchial cells are columnar in shape and arranged in pseudostratified manner. The progenitor population for this region is the **basal cell**, which gives rise to the ciliated cells and to a lesser extent the mucous cells (also referred to as goblet cells) (Warburton et al., 2010).

Early developmental studies in the hamster lung identified a basal cell population that did not reach this lumen in the newly formed pseudostratified epithelium by E13. This basal cell population was found to undergo a rapid increase in the rate of mitosis between E13 and E15 which resulted in expansion of the basal cells as well as the appearance of other cell types in this region (McDowell et al., 1985). More recent studies have determined some of the candidate genes marking this early basal cell population. As early as E10.5, p63 expressing cells can be found in the tracheal epithelium. P63 is a homologue of p53 with profound impact on

development since p63-null mice died shortly after birth and lack limbs, epidermis as well as epithelial development (Mills et al., 1999; Yang et al., 1999). In the lung, cells in the trachobronchial epithelium expressing p63 represent the progenitor population of basal cells. By E15.5 these cells have differentiated selectively into **ciliated cells** that lack p63 expression and are now identifiable by  $\beta$ -tubulin-IV expression. Later in development, the basal progenitor population becomes lineage restricted to a cyokeratin 14/cytokeratin 5 population while retaining p63 expression. This suggests a role for p63 in committing early stem cell populations in the trachea into basal cell progeny and the future maintenance of the mature basal cell population (Daniely et al., 2004).

While absence of p63 expression results in specification of ciliated cells, the forkhead transcription factor Foxj1 plays a role in maturation and maintenance of these cells. Targeted deletion of *Foxj1* in the respiratory epithelium resulted in defective ciliogenesis and randomized left-right asymmetry resulting in a disruption of the mucocilliary elevator (Brody et al., 2000). Notch signaling affects the balance of ciliated cells to secretory Clara cells, with absence of Notch1 resulting in an increase in ciliated cells at the expense of the secretory lineage (Guseh et al., 2009; Morimoto et al., 2010). Transgenic expression of the Notch pathway in the embryonic airways results in an increase in **mucous cells** at the expense of the ciliated cell lineage, while pharmacological inhibition of Notch led to a block of differentiation to mucous cells in lung explants (Guseh et al., 2009). Mucous cells play an important role in airway remodelling in the context of lung disease. *Spdef*, an ETS domain transcription factor is expressed in mucous cells in the respiratory tract and the gut. Overexpression of this factor in a subset of Clara cells resulted in differentiation to the mucous cell lineage without proliferation of the cells and loss-of-function experiments demonstrated that *Spdef* is necessary for mucous cell metaplasia in response to pro-inflammatory cytokines (Chen et al., 2009). *Foxa2* expression has also been shown to play a role in the differentiation to the mucous cell lineage, as targeted deletion of this transcription factor led to an increase in mucous cells. Conversely, overexpression of *Foxa2* resulted in a block of the mucous cell metaplasia in mouse models of allergic asthma (Park et al., 2009). These data demonstrate the complex interplay between multiple signaling pathways and environmental stressors necessary for differentiation to the mucous cell lineage. At approximately E17 in the mouse, or 13 weeks in the humans, mature ciliated columnar cells are present and mucous secreting cells can be detected. These mucous secretions work with the ciliated cells to help clear the airway and keep the upper airways moist.

Submucosal glands are located just below the surface of the epithelium in the cartilaginous airway and connect to the epithelium through a complex system of ducts and tubules. The serous cells within these glands produce

antibacterial factors (i.e., lysozyme), while the mucous cells produce mucous and fluid in response to airway infection and irritation (Liu and Engelhardt, 2008). Located next to the submucosal glands are the **pulmonary neuroendocrine cells (PNEC)**. This is the first fully differentiated epithelial cell type in the murine airway (E16) and appears in the human airway by 10 weeks gestation. These cells can be found alone or in groups that are innervated (referred to as neuroendocrine bodies). During fetal life, these cells secrete gastrin-releasing peptide and calcitonin-gene related peptide which is thought to help direct growth and differentiation of the lung (Hoyt et al., 1991; Sunday et al., 1993). At E13 in the mouse, subsets of epithelial cells begin to express the Mash1 transcription factor, which can persist or be turned off by Notch signaling mediated through Hes1 in surrounding cells. Cells that retain Mash1 are committed to the PNEC lineage, while cells that down regulate it differentiate to the **Clara cell** lineage. Evidence to support this hypothesis comes from *Hes1* null mutants which have an increase in PNEC at the loss of Clara cells (Ito et al., 2000).

Currently, PNEC lineage commitment is the best understood in terms of transcriptional mediators and signaling. Determinants of other lineages are limited by available markers for differentiation and commitment in the lung epithelium (Rawlins, 2008). Clara cells are present in the bronchiolar epithelium and emerge during the 19th week of human gestation (approximately E16.5–17 in the mouse lung). In the proximal epithelium they are a rare cell population, but as you move into the bronchiolar epithelium, Clara cells can make up as much as 80% of the population. These cells express Clara cell secretory protein (CCSP), which is the most abundant secretory protein found in the surface fluid of the airway (Wong et al., 2009). Lineage tracking studies have found that most Notch-activated cells differentiate into the Clara cell lineage, with a few ciliated cells making up the remainder (Morimoto et al., 2010).

It has been proposed that secretoglobin Scgb1A1 positive cells found in mature Clara cells of the proximal airway are derived from the more primitive Scgb3A2 precursors (Reynolds et al., 2002). Conditional endoderm knockout studies using the canonical Notch pathway have provided further evidence for this hypothesis and have proven that it is a Notch-dependent process (Morimoto et al., 2010; Tsao et al., 2009). Evidence for various subtypes of Clara cells with different functions and differentiation capacity has come from injury models in mature lungs and will be further discussed later. To date, the mechanisms regulating fate decisions and markers of these subtypes have yet to be determined.

The distal lung is the last area of the lung to differentiate to its functional adult form and does so in conjunction with the vasculature and surrounding mesenchyme (discussed below). Early developmental studies in the rat lung found that between E18–20 (roughly E16–E18 in the mouse), the distal

lung was composed of glycogen-rich cuboidal **alveolar type II cells**. By E20 lamellar bodies, the intracellular storage organelles of surfactant can be seen and at E21 the first histological evidence of the flattened **alveolar type I cells** is visible. Between E21 and postnatal life, an intermediate cell type was observed, which gave way to the normal distribution of the postnatal lung after birth (Adamson and Bowden, 1975). This study found that DNA synthesis was occurring at a high rate in the type II cells and was relatively rare in the type I cells. These results were used to establish that type II cells were a progenitor for type I cells. Rigorous modern lineage tracing *in vivo* is needed to add further evidence to this generally accepted hypothesis.

In the adult lung the type II cells make up less than 5% of the total surface area but account for 60% of the alveolar epithelial cell population. The mature type II cells can be identified by their expression of surfactant proteins (SP) A, B, C and D. SP-A and -D play a role in host defence, while SP-B and -C contribute to the physical properties of the surfactant. Type I cells compose 95% of the surface area and account for 40% of the alveolar cell population (Whitsett et al., 2002). T1 $\alpha$ , a developmentally regulated apical membrane protein, is expressed in the epithelium throughout lung development but later becomes restricted to mature type I cells. Mice null for T1 $\alpha$  are phenotypically normal until E18.5, when the absence of T1 $\alpha$  blocks differentiation into type I cells and results in death at birth due to lungs that cannot inflate (Millien et al., 2006). This study suggests an important role for T1 $\alpha$  in the differentiation to type I cells, but the mechanism of action has yet to be characterized. A distinction between the conducting airway and the respiratory airway is the involvement of the Notch pathway for cell fate determination. As discussed above, Notch signaling plays an important role in cell fate decisions in the upper airway but loss of function studies have demonstrated that Notch does not play a role in the maturation of the distal lung (Morimoto et al., 2010; Tsao et al., 2009).

## ADULT LUNG

The adult lung is maintained throughout life by the balance of proliferation and differentiation of tissue specific stem and progenitor cells that work together to replace dead or injured cells. Within the adult lung, various trophic units or niches exist that harbour the adult progenitor cells. Given that the lung is an organ with relatively slow turnover, injury models have been applied to determine which stem cells within a niche are able to give rise to the surrounding cell types. The slow cycling property of the stem cells makes it possible to identify candidate cells using DNA labelling techniques such as BrdU labelling. These cells are commonly referred to as label retaining cells (LRC). This allows cells with limited differentiation capacity to be identified as cells contributing to the entire trophic unit and be

defined as stem cells. While this criterion cannot be used alone in identifying this population, it has been useful in narrowing down the search within the niches of the lung (Borthwick et al., 2001; Liu and Engelhardt, 2008b).

## **Adult Epithelial Stem and Progenitor Cells**

By the time alveolarization and microvascular maturation is complete the airway is fully differentiated with the exception of a few progenitor cell populations located within specific regions of the lungs. These specialized niches allow the appropriate microenvironment for maintenance and also targeted differentiation and migration of the cells when necessary (i.e., after injury). Candidate supporting features within these regions include matrix factors, cytokines, and growth factors from neighbouring cells (Watt and Hogan, 2000).

### **Upper Trachea (Submucosal Glands)**

In humans, submucosal glands are found in abundance from the proximal trachea until the 10th generation airway and are rich in goblet cells (Wine and Joo, 2004). In contrast, submucosal glands in rodents are found only in the larynx and proximal trachea, with Clara cells taking over the function of the goblet cells in the lower regions of the lung (Plopper et al., 1980; Widdicombe et al., 2001). This discrepancy in location and cell composition has led to the use of model species such as ferrets instead of rodents, given their similarities to the human airway.

Mouse trachea studies using repetitive SO<sub>2</sub> inhalation, a well-documented model for pulmonary epithelial cell injury (Lamb and Reid, 1968), as well as continuous BrdU labelling showed that at 6 days after the last injury, densely stained cells could be found in the gland ducts and also scattered along the trachea. By 21 and 95 days post-injury, LRC were localized preferentially to the submucosal glands in the upper trachea, and at the cartilage-intercartilage junction of the lower trachea (Borthwick et al., 2001). To determine if the population of cells within the glands is able to regenerate the surface epithelium of the upper trachea, protease-treated tracheas were transplanted into T-cell deficient mice to assess re-growth. By day 28, cells had repopulated the surface of the denuded trachea, suggesting that cells residing in the glands were responsible for the recellularization of the surface epithelium (Borthwick et al., 2001). While the positioning of this renewing cell type seemed to suggest PNEC, dual staining did not co-localize calcitonin-gene related protein, a PNEC marker, with LRC. There were many more LRC than PNEC positive cells although in the distal trachea they were often found in close proximity to one another (Borthwick et al., 2001).



## **Lower Trachea/Bronchiolus**

There appear to be multiple cell types responsible for maintaining the pseudostratified epithelium of lung that appear to be distinct from the cell types that maintain the simple epithelium of the bronchioles (Evans et al., 1978; Evans et al., 1986). Both basal cells and Clara cells have been proposed as the multipotent progenitor type in the adult proximal lung. One of the functions of Clara cells in the airway is to metabolize lipophilic compounds through cytochrome P450-mediated oxidation, which makes these cells selectively susceptible to injury by these chemicals (Boyd, 1977). Variant Clara cells were identified after the administration of naphthalene, a Clara cell-specific cytotoxicant, to the lungs of adult mice (Stripp et al., 1995). This study, and many others since, identified a subset of Clara cells that were immune to the injury and that was responsible to the repopulation of the damaged epithelium after injury. These variant Clara cells were found to be preferentially located at branching points in the lung (Stripp et al., 1995). Targeted injury to the ciliated cells using NO<sub>2</sub> demonstrated that the Clara cells rather than the basal cells were able to divide and differentiate into cells from the ciliated, serous and goblet cell lineage (Evans et al., 1986). Seemingly in opposition to this finding, other groups sorting for the basal cell markers Griffonia simplicifolia I lectin and cytokeratin 14 found that these cells engrafted and de-differentiated to a similar degree as Clara cells and contributed to all lineages in a denuded trachea injury model (Liu et al., 1994). In an attempt to reconcile these finding, two different injury models were used to determine the contribution from each of these lineages. One model involved ablation of all but the variant Clara cells using naphthalene, while the other involved the preferential ablation of all Clara cells in a transgenic mouse expressing thymidine kinase in CCSP-expressing cells by ganciclovir. In the naphthalene injury, Clara cells were the primary cell type to re-populate the injured airway surface as has been previously reported (Evans et al., 1975). In the Clara cell-ablated model, it was the GSI+/cytokeratin 14+ population that was recruited to repopulate the damaged lung (Hong et al., 2004a; Hong et al., 2004b). From these experiments it was proposed that while Clara cells were the major progenitor cell of the bronchial epithelium, basal cells represented a secondary multipotent progenitor cell in the event of loss of the Clara cell population (Hong et al., 2004a). The finding of a secondary progenitor population in the upper airway that act in a hierarchical manner has implications for various diseases where remodelling occurs. It has yet to be determined if the epithelium regenerated from the basal cells is functionally the same as that derived from the preferential cell source, and if this has an impact on future responses to injury (Hong et al., 2004a).

In contrast to the tracheobronchial pseudostratified columnar epithelium, the bronchiolar airways are lined with a simple columnar to cuboidal epithelium. This region is composed primarily of Clara cells with some ciliated cells and sparsely distributed PNEC either alone or in NEB. Within the NEB, two populations of label retaining cells have been identified and shown to proliferate following chemical ablation, PNEC expressing CGRP and a population of variant Clara cells. All Clara cells were ablated by ganciclovir using a transgenic mouse expressing thymidine kinase in CCSP-expressing cells to determine if the CGRP cells had multipotent progenitor properties. While CGRP cells were able to proliferate they were unable to repopulate the Clara cell-depleted lung suggesting a role in the maintenance of a stem cell niche, but not a multipotent progenitor (Hong et al., 2001). Naphthalene injury demonstrated that the label-retaining variant Clara cells were able to proliferate as well as differentiate to a variety of lineages and repopulate the injured lung. These studies support the belief that the variant Clara cells are the progenitor population of the bronchiolar region of the lung (Hong et al., 2001).

### **Terminal Bronchioles (Bronchoalveolar duct junction (BADJ))**

Studies identifying the progenitor population of the conducting airway lead to an interest in the cell type that repopulates the terminal bronchioles and alveolar region following injury. Naphthalene resistant variant Clara cells were found to be localized to the bronchoalveolar duct junction (BADJ) region and contained a subset of LRC, suggesting stem cell properties. These cells were found to contribute to the repopulation of the terminal bronchioles following injury and were not associated with NEB. These data suggest that a distinct population of progenitors for this region exists to replace the terminal bronchioles following Clara cell injury (Giangreco et al., 2002). A more recent and controversial study identified a subset of variant Clara cells at the BADJ that are CCSP+, SPC+ and CGRP-. These were proposed to be bronchoalveolar stem cells (or BASC) that can contribute to the repopulation of not only the terminal bronchioles but also the alveolar compartment (Kim et al., 2005). Evidence for this comes from the observation that these cells divide first in response to naphthalene or bleomycin injury in the mouse and that the population can self-renew and differentiate to bronchiolar and alveolar lineages *in vitro*. The authors also demonstrated that these cells expanded in response to K-ras in culture and *in vivo* (Kim et al., 2005). Many of the criticisms of this paper revolved around the lack of *in vivo* evidence that this cell population was biologically relevant. Both Clara cells and type II cells have been shown to proliferate in response to naphthalene or bleomycin injury and cell behaviour *in vitro* cannot always be extrapolated to *in vivo* (Rawlins, 2008).

Independent studies have shown that deletion of p38 $\alpha$  MAP kinase in the adult lung results in an increase in the dual positive population, as well as making it more susceptible to activated Ras-mediated oncogenic transformation (Ventura et al., 2007). The deletion of p38 $\alpha$  MAP kinase also resulted in an increase in proliferation and defective differentiation of the lung leading to an immature and highly proliferative epithelium. This supports the idea that the CCSP+ /SPC+ population is an immature transient population that is particularly susceptible to oncogenic transformation (Rawlins, 2008). Lineage tracing studies over the course of lung development using a CCSP+-CreER knock-in system in reporter mice allowed the *in vivo* tracking of all CCSP-expressing cells, including the dual CCSP/SPC expressing subset identified by Kim (Kim et al., 2005). The results suggested that while many CCSP-expressing cells act as long term progenitors in the bronchioles, they do not contribute significantly to the alveolar compartment (Rawlins et al., 2009). Furthermore they determined that the dual CCSP/SPC expressing population did not have any special function during development, postnatal growth, adult homeostasis or in lung injury models (Rawlins et al., 2009). These findings support the traditionally held view that a separate progenitor populations exist within each region of the lung and act locally for maintenance and repair.

## **Alveolar Region**

Classic lineage tracing studies provide evidence that in adult lungs, type II cells can differentiate into a type I phenotype. Following NO<sub>2</sub> injury in rats, type II cells were labelled with tritiated thymidine and identified 14 days later by electron microscopy. It was found that type II cells lost their label in conjunction with large expansion of the type I population, suggesting that the type II cells were differentiating into type I cells (Evans et al., 1975). More modern studies have sought to further specify the subset of type II cells that are multipotent (Reddy et al., 2004). Type II cells are known to be sensitive to hyperoxia (> 90% oxygen), causing rapid cell division as well as apoptosis and increase in telomerase activity (a hallmark of progenitor cells) in a subset of cells (Buckley et al., 1998; Bui et al., 1995; Driscoll et al., 2000). In order to further elucidate this progenitor population of type II cells, hyperoxia damaged and normal steady-state lungs were compared for a variety of different markers. Reddy et al. found an E-cadherin negative type II population that had low levels of DNA damage in response to hyperoxia as well as high levels of telomerase activity. While further characterization of this population need to be done, the initial data suggest that this subpopulation has the characteristics of an adult progenitor cells and may

be the population responsible to regeneration in the alveoli (Reddy et al., 2004) (see Tables 1 and 2 for a summary of embryonic and adult progenitor lineages in the lung).

**Table 1.** Summary of embryonic progenitor cells in the lung epithelium.

| Embryonic Progenitors                    | Daughter cells                                                                        | Reference                |
|------------------------------------------|---------------------------------------------------------------------------------------|--------------------------|
| Basal Cell                               | Ciliated cells<br>Mucous cells                                                        | (Warburton et al., 2010) |
| PNEC                                     | Clara cells<br>Variant Clara cells                                                    | (Ito et al., 2000)       |
| Alveolar type II cell                    | Alveolar type I cells                                                                 | (Reddy et al., 2004)     |
| Id2 <sup>+</sup> (pseudoglandular phase) | Clara cells<br>Ciliated cells<br>Mucous cells<br>PNEC<br>Alveolar type I and II cells | (Rawlins et al., 2009a)  |
| Id2 <sup>+</sup> (canalicular phase)     | Alveolar type I and II cells                                                          | (Rawlins et al., 2009a)  |

**Table 2.** Summary of adult progenitor cells in the lung epithelium.

| Adult Progenitors                                    | Daughter cells                                                | Reference                 |
|------------------------------------------------------|---------------------------------------------------------------|---------------------------|
| Submucosal gland progenitor cell (unknown cell type) | Immature cuboidal epithelium of unknown cell type             | (Borthwick et al., 2001a) |
| Variant Clara cells                                  | Pseudostratified epithelium of trachea                        | (Hong et al., 2004a)      |
| Basal cells                                          | Pseudostratified epithelium of trachea if Clara cells damaged | (Hong et al., 2004a)      |
| Alveolar type II cells                               | Alveolar type I cells                                         | (Reddy et al., 2004)      |

## MESODERM PROGENITOR CELLS

As discussed earlier the airways are predominately derived from the endoderm. The lung however, is a complex and intricate organ that also requires substantial contribution from cells and tissues derived from the mesoderm germ layer. Three important tissues of mesoderm origin contribute to pulmonary function and sustenance in 3 distinct ways: 1) the pulmonary vasculature provides vessels through which blood flows for gas exchange with tissues and the environment, 2) the pulmonary vascular and airway smooth muscle regulates the diameter of the larger blood vessels and airways, respectively, and 3) the pulmonary immune system provides a cellular defensive mechanism to maintain healthy and clear airways free of inflammation or infection. Mesoderm-derived cells therefore contribute substantially to the structural and functional properties

of the lung. These lung cells and their progenitors residing in this layer are referred to collectively as lung mesenchyme. In contrast to the epithelial lineage, very little is known about the pulmonary mesodermal progenitors in adult lung tissue, although some efforts have been made in studying these progenitors in embryonic and fetal lungs. To further complicate this, it is still unknown what are the numbers, nature and characteristics of the mesoderm-derived cells found in the adult or developing lung and thus ligand marker characterization yields limited information. To underscore this, a recent study illustrated that no unique ligand or molecular marker sufficiently and uniquely identified any one particular adult pulmonary mesoderm-derived cell. Without this information, identification of general pulmonary mesodermal progenitor cells becomes very challenging (Rawlins, 2011).

### **Pulmonary Mesodermal Progenitors**

Fate mapping is an accepted technique established in the first half of the 20th Century and subsequently mastered over the last 30 years that is used to determine the cellular and developmental sequences that occur during and beyond embryogenesis. Most fate mapping endeavours trace the cells of early development to their later cellular successors using fluorescent labelling. This allows for the visual identification of specific cellular precursors and with detailed information about their respective developmental history and future developmental fate (Dale and Slack, 1987). For example, recently this powerful technique of fate mapping with fluorescent reporter transgenes has been used to specifically identify when, where and from what tissues haematopoietic stem cells arise (Bertrand et al., 2010; Bertrand et al., 2007). The analysis of growth factor secretion, growth factor receptor expression and the developmental changes identified by fate mapping clarify and further substantiate the developmental milestones of particular tissues.

During murine lung development, populations of mesenchymal cells have been identified as early as day E12.5 (White et al., 2006). This appears to coincide with *Fgf10* expression localized in the submesothelial mesenchyme, the region of mesenchymal tissue in direct contact with the mesothelium (membrane lining the pleural cavity) and corresponds to active branching morphogenesis in distal lung development (Bellusci et al., 1997). The sonic hedgehog receptor *Ptch1* (patched 1) may also regulate branching morphogenesis but its expression is characteristic of more general distal lung mesenchyme (Pepicelli et al., 1998; Weaver et al., 2003). These observations suggest that although cells derived from similar germ layers, even within the same organ, respond to growth factors and molecular markers in varying ways with different expression patterns directing

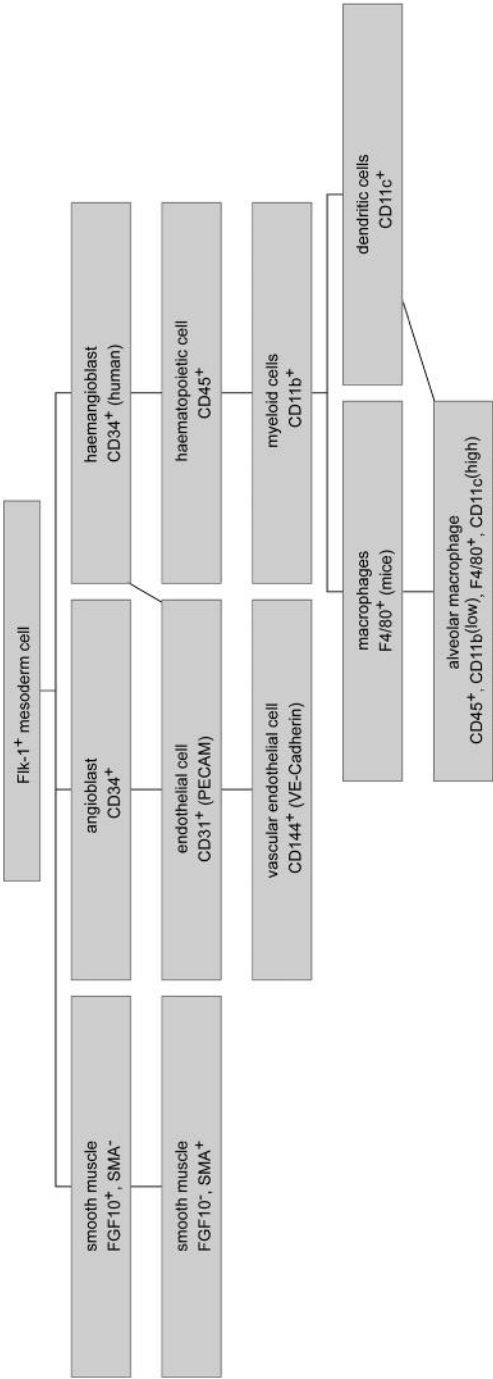
targeted changes, thus promoting very local signals to establish specified cellular fate and localized tissue modeling. Embryonic lung research has been successful in identifying many of the molecular markers, growth and transcription factors that promote mesenchymal proliferation and expansion rather than specific progenitor populations. In addition to the embryonic roles of *Ptch1* and *Fgf10* highlighted above, *Fgf9* has been identified as a major factor involved in lung mesenchymal progenitor proliferation during the pseudoglandular stage of lung development by eliciting a Wnt signaling loop and signaling through the *Fgfr1* and *Fgfr2* receptors (Rawlins, 2011). To date, however, no one specific lung mesoderm stem cell or progenitor has been identified or characterized that has the potential to become lung vascular endothelial cells, lung smooth muscle cells, or lung haematopoietic cells. Chart 1 summarizes the cellular progenitors, their progeny and the ligand markers of mesodermal cells involved in lung development.

## Pulmonary Vasculature

The pulmonary vasculature can contain kilometers of vessels and capillaries providing circulation to the lung and to the alveolar structures of the lung for efficient gas exchange with the circulating blood volume. Angiogenesis and vasculogenesis are the two mechanisms by which lung vasculature may develop. During angiogenesis, vessels sprout from pre-existing vessels and extend the already established vasculature. Conversely, vasculogenesis describes vessels arising spontaneously from blood vessel precursor cells called angioblasts, or more recently simply termed EPC, for endothelial progenitor cell.

Differences arising from historical definitions of the angioblast and EPC have recently stirred some controversy, as the criteria used to describe the vascular cell progenitor population, which may include, for example, the presence of the ligand marker CD34, has not been universally accepted (Kovacic et al., 2008). Some evidence exists suggesting that during embryonic lung development in mice, vessels containing endothelial cells that express the endothelial marker CD31 (PECAM), are present at E9.5–10, suggesting that these cells and vessels sprouted from pre-existing circulation by angiogenesis, rather than arising *de novo* from a *Flk-1*+/*CD34*+ angioblast (Parera et al., 2005). Furthermore, another study showed evidence for angiogenesis by tracing embryonic lung vessels to general circulation during the pseudoglandular stage and illustrating that the vessels actively extend at their tips (Schwarz et al., 2009). In a different study, evidence for vasculogenesis during the pseudoglandular phase was also shown by illustrating that *Flk-1* vascular progenitors were present in the E10.5 embryonic lung and could generate vessels (Schachtner et al., 2000; Yamamoto et al., 2007). Thus, there appears to be evidence for both vessel

Chart 1. Mesoderm-derived cells of the lungs



formation mechanisms to be active in pulmonary development and it is likely that local cues help to regulate the balance in favour of one mechanism over the other at varying times and locations.

These highlighted studies focused mostly on embryonic lung development but to fully appreciate pulmonary vascular development and the remodeling potential of the lung the vascular progenitors available in adult lung tissue need to be determined. To investigate the origin and fate of pulmonary endothelial cells, Lin and colleagues performed left lung pneumonectomies and studied the gene expression of the right lung during the subsequent compensatory lung growth. Specifically, the right lung was enzymatically digested and CD31 endothelial cells isolated by FACS at selected days post pneumonectomy. The gene expression of these cells was analyzed using real-time quantitative PCR. More than 20 angiogenesis-associated genes displayed significantly increased expression at multiple time points. The expression profile was evaluated using cluster analysis and it revealed that angiogenic gene expression in compensatory lung growth occurs in four phases, first by a wave of downregulation followed by 3 waves of upregulation (Lin et al., 2011). These findings establish a gene expression network that appears to regulate angiogenesis in the lungs; however, the study does not indicate the source of the CD31 cells and from what progenitor cells they arise.

Vascular endothelial cells may arise from a series of progenitors expressing identifiable markers. For example, mesoderm-derived cells express Flk-1, while endothelial progenitors express CD34. Endothelial cells express CD31, which is commonly named platelet-endothelial cell adhesion molecule (PECAM). Endothelial cells can develop into vessels for the lymphatic or the vascular system. Identification of vascular endothelial cells is achieved by CD31 surface expression in conjunction with the vascular marker CD144 (VE-Cadherin). To determine the source of endothelial progenitors in the lungs, Chamoto and colleagues also used the pneumonectomy-induced compensatory lung growth model. Instead of investigating only wild-type mice, they surgically joined the right side of GFP mice to the left side of wild-type mice from forelimb to hindlimb, thereby establishing a closed common blood circulation of paired 'parabiont' mice. They surgically removed the entire left lung of the wild-type mouse creating a series of parabiont animals with only 3 lungs: 2 from the GFP parabiont and 1 from the wild-type parabiont counterpart. The common circulation allowed for the transfer of GFP+ cells to move freely within the wild-type mouse. At preselected timepoints subsequent to the pneumonectomy, the mice were sacrificed and the remaining right lung of the wild-type parabiont counterpart was analyzed. The CD31+ pulmonary endothelial cell population from the wild-type right lung was quantified by flow cytometry and displayed a greater than 3-fold population increase.



This suggested to the authors that endothelial progenitors were contributing to the compensatory lung growth. The source of the progenitors was then established by quantifying the CD34 population in the compensatory lungs.

First it was shown that the CD34 endothelial cell population in the compensatory lungs of the wild-type parabiont counterpart increased greater than 7-fold and nearly 20% of these cells were actively proliferating in the lung. In particular, more than two thirds of the CD34<sup>+</sup> endothelial progenitor cells found in the compensatory lung of the wild-type counterpart mice expressed GFP. Since the GFP<sup>+</sup>/CD34<sup>+</sup> cells could only come from the GFP counterpart of the parabiont pair the CD34 endothelial progenitors must have been derived from peripheral blood. In total, nearly 75% of all CD34 lung endothelial progenitors came from the peripheral blood and were effectively incorporated into the lung vasculature. Moreover, these blood-borne CD34 endothelial cells and progenitors displayed increased transcriptional and mitotic activity compared to the CD34<sup>-</sup> endothelial cells. Thus, the peripheral blood appears to be a critical source of actively dividing CD34<sup>+</sup> endothelial progenitors for the lungs (Chamoto et al., 2012).

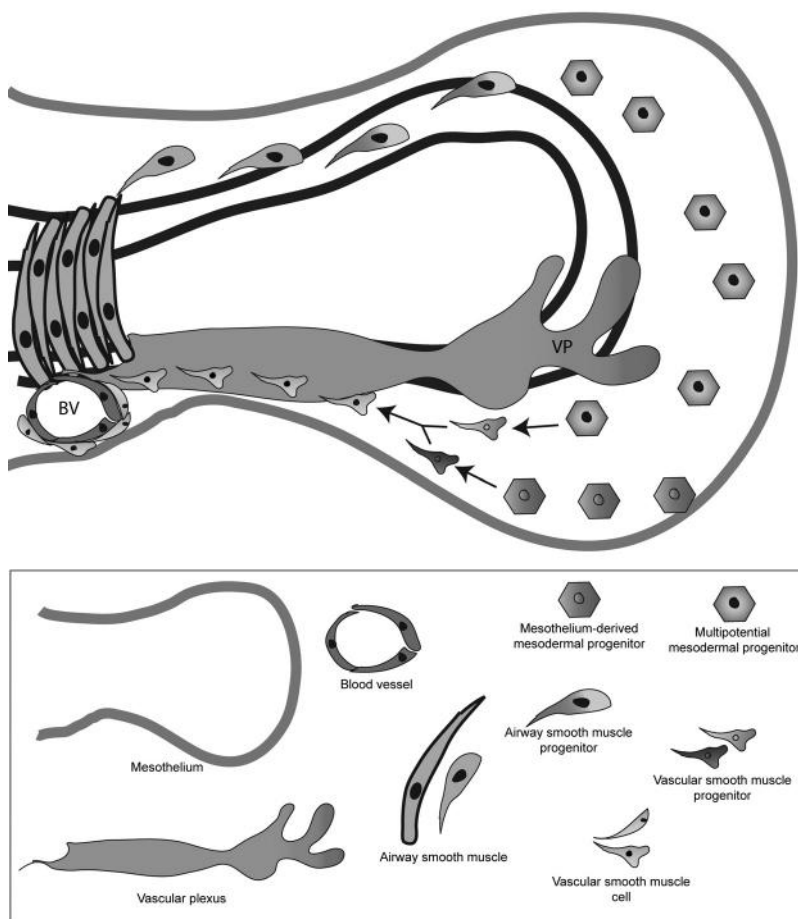
It is unclear what is the source of the remaining CD34<sup>-</sup> endothelial cells and there is conflicting evidence from a previous study suggesting that circulating endothelial precursors do not contribute to compensatory microvascular lung growth (Voswinckel et al., 2003). However, another study investigating the pulmonary microvasculature in rats found that some endothelial cells display highly proliferative capacity suggesting the ability to self-renew. Furthermore, some of these cells were characterized as resident microvascular endothelial progenitors expressing the endothelial markers CD31, CD144, eNOS, and von Willebrand factor. These cells displayed rapid vasculogenic capacity both *in vitro* and *in vivo* (Alvarez et al., 2008). In mice, it was further established that in addition to having a population of mature lung microvascular endothelial cells, a local microvascular endothelial progenitor cell also resided in the adult lungs that could give rise to both lymphatic (CD31<sup>+</sup>, Lyve1<sup>+</sup> and Prox1<sup>+</sup>) and vascular (CD31<sup>+</sup>, Lyve<sup>-</sup>, Prox1<sup>-</sup>) vessel forming endothelial cells; with these vascular progenitors giving rise to cells expressing endothelial markers such as CD102 (ICAM-2), CD105 (endoglin) and CD144 (VE-Cadherin) (Schniedermann et al., 2010). In contrast to these studies suggesting that lung endothelial cells arise from circulating progenitors, a current study recently cast doubt on the origins and existence of pulmonary endothelial cell progenitors. In their study, Ohle and colleagues (Ohle et al., 2012) used a model of hypoxic lung injury in mice and then evaluated the maintenance and repair of the lung endothelium by using transplanted haematopoietic stem cells or whole bone marrow-derived cells. They found little evidence of marrow-derived cells contributing to the regeneration of

the pulmonary endothelium, but stopped short of detailing what cells do contribute this vascular regeneration and only suggested that regenerated lung endothelium arises from previously existing vascular cells (Ohle et al., 2012). Despite conflicting discussions and studies, it is becoming clearer where the pulmonary vascular endothelial progenitors originate and reside and how they function, but further information concerning the nature of the lung environment and its contribution to pulmonary vascular expansion and maintenance is still necessary.

## **Pulmonary Smooth Muscle Tissue**

Smooth muscle (SM) tissue derived from the mesoderm can be located in the lungs in two major areas: 1) airway smooth muscle (ASM) and 2) vascular smooth muscle (VSM). Airway smooth muscle regulates the diameter of the airway conduits and overactive smooth muscle or hyperproliferative ASM cells may contribute to diseases such as chronic asthma by constricting airway flow unnecessarily. Similarly, over-activity of pulmonary VSM may contribute to pulmonary hypertension (Morrisey and Hogan, 2010). Thus, it is important to understand the progenitor cells that will develop into pulmonary SM. This is an area of lung biology that is poorly described and largely unknown; however, some hypotheses do exist that explain the smooth muscle development of the lungs. Figure 3 highlights the putative progenitors of pulmonary vascular and airway smooth muscle and their progeny during the pseudoglandular phase of lung development.

To determine more precisely the nature of ASM progenitor cells, lineage tracing studies have been employed extensively showing that airway smooth muscle may originate from peripheral mesenchyme progenitors that express Fgf10. These cells migrate along the extending peripheral airway, like a sleeve extending down one's arm, and begin expressing  $\alpha$ -smooth muscle actin in response to Shh and Bmp4 signals very early in development (Warburton et al., 2008). To maintain a pool of SM progenitors in the distal lung, Fgf9 from endoderm and mesothelium complements signals from Fgf10 (Morrisey and Hogan, 2010). Distal mesenchyme cells that express Fgf10 but not  $\alpha$ -smooth muscle actin give rise to  $\alpha$ -smooth muscle expressing, Fgf10-negative, parabronchiolar smooth muscle. Furthermore, there is speculation that some proximal mesenchymal cells located close to the trachea and bronchi can give rise to distinctively parabronchiolar smooth muscle cells. This evidence suggests that two SM progenitor populations may exist: a distal tip population and a proximal bronchi population (Rawlins, 2011). Further examination of these details is necessary to clearly understand the relevance, role and dominant locations of the SM progenitors in the lungs.



**Figure 3. Mesoderm-derived progenitors during the pseudoglandular phase of lung development.** At the distal tip multipotential mesodermal progenitors (grey hexagons) can give rise to both vascular smooth muscle progenitors (brown hexagons) near the vascular plexus (VP) or airway smooth muscle progenitors (brown & grey drops; upper portion of the figure). Fully differentiated vascular smooth muscle cells (green) arise from either mesothelium (brown hexagons) or mesodermal progenitors. These differentiated vascular smooth muscle cells surround the blood vessels (BV). Alternatively, mesodermal progenitors give rise to airway smooth muscle progenitors (top). Progenitors develop into the airway smooth muscle cells (brown vertical elongated structures) surrounding the developing airway.

*Color image of this figure appears in the color plate section at the end of the book.*

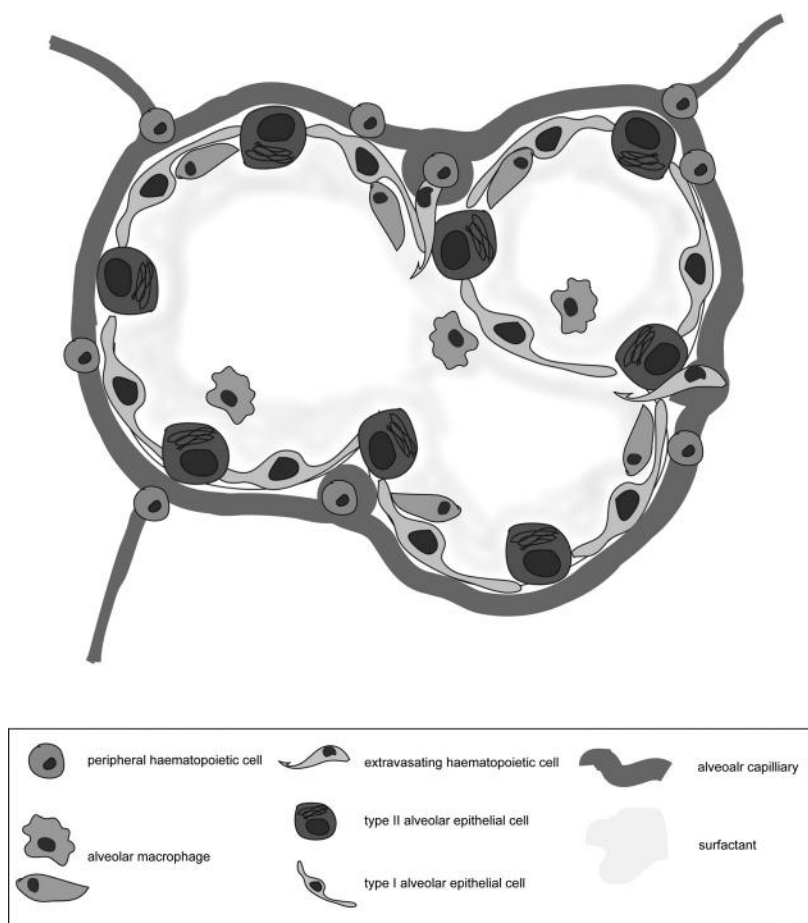
The nature and development of pulmonary VSM is also poorly described. Current lineage-tracing studies indicate that about a third of pulmonary VSM derives from the mesothelium (Que et al., 2008). Closer examination of the pulmonary endothelium has revealed that the proportion

of endothelial-derived VSM can differ significantly between proximal and distal locations of the developing lung; specifically, VSM of the proximal vessels predominately originate from endothelium, whereas very little endothelial contribution is noted for the more distal VSM cells of the lungs (Morimoto et al., 2010). Curiously, the mesothelium- and endothelium-derived SM progenitors only contribute to approximately two thirds of the VSM identified in the lungs (Rawlins, 2011). It remains unknown what contributes to the other one third of pulmonary VSM cells. Other models suggest that undifferentiated cells from the mesoderm begin to differentiate into SM when they surround the vascular endothelium; this likely results from a combination of signals originating locally from endothelial, epithelial and mesothelial cells (Morrisey and Hogan, 2010). To date, no consensus has been reached regarding the exact origins of the pulmonary VSM progenitors, and due to its implication in diseases, regenerative and transplantation medicine, further examination is warranted.

### **Pulmonary Hematopoietic Cells—The Alveolar Macrophage**

Early in embryonic development multipotent cells arise that are called haemangioblasts and act as precursors of both endothelial progenitors, or angioblasts, and hematopoietic stem cells (HSC) (Xiong, 2008). HSC are self-renewing, reside mostly in the bone marrow, and are the precursors of all blood cells. Their cellular progeny includes cells of the lymphoid lineage of blood cells, such as T-cells, B-cells, and Natural Killer (NK) cells and cells of the myeloid lineage of blood cells such as macrophages, dendritic cells, and red blood cells (Geissmann et al., 2010; Zhao et al., 2012). In the lungs, macrophages are the most populous and important hematopoietic cell that is continuously present. Macrophages are cells of the innate immune system that are considered specialized phagocytes. Their primary function is to engulf and clear dead or dying cells and pathogenic material (Aderem and Underhill, 1999). Two populations of macrophages exist in the lungs: interstitial tissue macrophages that function like macrophages in other tissues and reside in the areas surrounding the airways and specialized airway phagocytes called alveolar macrophages that reside within the alveoli and clear airborne and soluble pathogenic material (Crowell et al., 1992; Fels and Cohn, 1986).

These macrophages in the alveoli have a very distinct phenotype compared to tissue macrophages, likely because they are in contact with air and the lipophilic surfactant that coats the epithelial layer of the alveoli (Figure 4). This unique environment requires an adapted phenotype for the purpose of maintaining clear airways. Hence, the lung environment contributes significantly to the development alveolar macrophages (Guth et al., 2009). Murine macrophages can be identified by their predominant



**Figure 4. Macrophages in the alveolar space.** The alveoli are saccular structures that are highly vascularized (red) with close contact amongst the alveolar capillaries and the epithelial cells (pink & purple). Hematopoietic cells (blue) circulate freely in the blood and some may extravasate into the alveoli (turquoise) to develop locally into alveolar macrophages (orange). It is presumed that alveolar macrophages reside within the lipophilic surfactant layer (yellow) and are mobile on the epithelial layer, however, some cells may not be in direct contact with the epithelial cells.

*Color image of this figure appears in the color plate section at the end of the book.*

surface ligands CD45 (haematopoietic), CD11b (myeloid) and F4/80 (macrophage). These three ligand markers illustrate the developmental pathway a myeloid macrophage follows from its origin as a HSC. The alveolar macrophage, however, shares many characteristics with both a myeloid-derived antigen-presenting cell called a dendritic cell and the tissue macrophage. For example, murine alveolar macrophages highly

express CD11c, which is characteristic of a dendritic cells, while also expressing CD45 and F4/80 and actively performing phagocytosis and antigen presentation (Guth et al., 2009). These characteristics highlight the exclusive nature of alveolar macrophages and raise many questions surrounding their development and maturation.

How and when the alveolar macrophage arrives in the lungs and for how long it remains in the alveoli are questions that are only starting to be answered in recent years. It is clear that during lung inflammation, hematopoietic cells are recruited to the lungs to rectify damage caused by injury, dead cells, and inflammatory mediators (Medzhitov, 2008; Milik et al., 1997). Presumably, these recruited cells function in the lungs only temporarily as they do not display the typical alveolar macrophage phenotypes (Guth et al., 2009; Janssen et al., 2008).

Although airway macrophages arise from hematopoiesis, their rate of replacement within healthy lungs is far slower than originally thought. Previous estimates had suggested alveolar macrophages turnover rate within healthy lungs was in the range of 10 to 30 days. However, recent evidence, using irradiated mice with lead-shielded thoraxes, suggests that alveolar macrophages may reside within the lungs of mice for months, years or even to the life-span of the animal. Moreover, it would appear that bone marrow hematopoietic cells do not necessarily contribute significantly to alveolar macrophage expansion in the airways during steady-state conditions (Murphy et al., 2008). This observation further supports the existence of especially unique qualities of the lung environment. These data are further substantiated by a distinct study where compensatory lung growth was examined after pneumonectomy. In this study by Chamoto and colleagues (2011a), the dynamics of alveolar macrophage growth and development in the lungs of rodents was examined by removing one lung in a mouse and observing the compensatory activities of hematopoietic and peripheral blood cells in the remaining lung.

Wild-type and GFP mice were paired and parabiotically joined surgically with common circulation and blood flow (as described previously). The left lungs of the wild-type mice were removed and the right lungs were monitored and examined at various time points over the course of 3 weeks. Macrophage populations in most tissues and organs can be repopulated rapidly by recruited hematopoietic circulating peripheral blood monocytic cells, especially in models of inflammation. In this model of parabiotic pneumonectomy, where inflammation of the remaining lung is absent, but only airspace compensation is present, the alveolar macrophage population was monitored as the wild-type right lung underwent compensatory regeneration. Like other tissues and organs, it was expected that circulating peripheral blood monocytes would be recruited to the regenerating lung during the compensatory redevelopment, and this would be indicated by

high levels of GFP+ alveolar macrophages in the right lung of the wild-type mouse arising from the GFP mouse. Indeed, it was found that alveolar macrophages increased during the compensatory lung growth of the right lung and that these cells exhibited angiogenic gene transcription. However, the majority of expanded alveolar macrophages were GFP-. These data indicate that the compensatory expansion of alveolar macrophages was actually resulting from locally proliferating cells and not from recruited hematopoietic stem cells, myeloid stem cells, or even peripheral blood monocytes (Chamoto et al., 2011). Both studies (Murphy et al., 2008; Chamato et al., 2011) suggested that within the alveoli, macrophages remain in a locally proliferative phase during steady state. Thus, in early postnatal lung growth, it is reasonable to suggest that hematopoietic cells initially migrate to the lungs and due to the local environment in the airways, these cells remain within the alveoli and expand their population proportionally as the airway volume increases during normal growth. These new findings demonstrate convincingly that what was originally known about the major hematopoietic cells of the airways only partially describes their development and the role that stem cells play in their population maintenance.

## CONCLUSIONS AND FUTURE DIRECTIONS

As illustrated above, embryonic progenitors are distinct from the cell types that take over the role of maintenance and regeneration in the adult population. The roles of an embryonic progenitor are to respond to the environmental cues and have the ability to develop into a wide array of cells as the organ develops. In contrast, in adult tissues there does not appear to be a master regulator cell capable of differentiation into all lineages, and instead there are niche-specific multipotent cells with the ability to maintain the region they reside in. As demonstrated with the Clara and basal cells in the upper airway (Hong et al., 2004a) there may be some overlap in multipotent potential in order to ensure that the lung can withstand various types of injury. Further characterization of steady-state conditions in the adult lung using lineage tracing experiments is necessary and will provide valuable insights for the field of regenerative medicine. One of the major impediments to elucidating the origin of each cell lineage within the lung is the lack of cell-type specific markers for many of the cell types. Another confounding factor is that certain cell-specific markers later in development are ubiquitously expressed during embryonic development (such as T1 $\alpha$ ), making target choice for lineage tracing difficult. The slow turnover rate of the lung is another factor making it difficult to study progenitor cells in the adult lung. Injury to the airway is often necessary to induce differentiation

and while this approach has added valuable information to the field, there is some question as to whether an injured state is an accurate recapitulation of steady-state renewal (Crystal et al., 2008).

## **ABBREVIATIONS**

|               |                                                                      |
|---------------|----------------------------------------------------------------------|
| AM            | alveolar macrophage                                                  |
| ASM           | airway smooth muscle                                                 |
| BADJ          | bronchoalveolar duct junction                                        |
| BASC          | bronchoalveolar stem cell                                            |
| CCSP          | clara cell secretory protein                                         |
| CGRP          | calcitonin gene-related protein                                      |
| eNOS          | endothelial nitric oxide synthase                                    |
| EPC           | endothelial progenitor cell                                          |
| F4/80         | murine macrophage marker                                             |
| Flk-1         | fetal liver kinase-1 (vascular endothelial growth factor receptor 2) |
| Fox           | Forkhead box protein                                                 |
| Gata          | GATA binding protein                                                 |
| GSI           | Griffonia simplicifolia I                                            |
| ICAM-2        | intercellular cell adhesion molecule 2                               |
| Id2           | Inhibitor of differentiation 2                                       |
| LRC           | Label retaining cell                                                 |
| Lyve-1        | lymphatic endothelial marker                                         |
| NEB           | Neuroepithelial bodies                                               |
| Nmyc          |                                                                      |
| PBMC          | peripheral blood mononuclear cells                                   |
| PECAM         | platelet endothelial cell adhesion molecule (CD31)                   |
| PNEC          | Pulmonary neuroendocrine cell                                        |
| Prox1         | lymphatic associated transcription factor                            |
| Ptch1         | Patched 1—a sonic hedgehog receptor                                  |
| Scgb          | Secretoglobin                                                        |
| Sox           | SRY (sex determining region Y) box                                   |
| SP            | surfactant protein                                                   |
| Spdef         | SAM pointed domain-containing Ets transcription factor               |
| Ttf1 (Nkx2.1) | thyroid transcription factor 1                                       |
| VE-Cadherin   | vascular endothelial cadherin (CD144)                                |
| VSM           | vascular smooth muscle                                               |



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# Stem Cells During Tooth Development

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## INTRODUCTION

The tooth is an excellent model organ to follow epithelial—mesenchymal interactions and understand the formation of complex morphology. It is also emerging as an useful model for studying how stem cells are regulated during development, and how adult stem cells contribute to tissue-cell turnover and repair.

Mammalian teeth are derived from ectoderm and neural crest (Chai et al., 2000; Rothova et al., 2012) with an invasion of vasculature, endothelial cells and pericytes, and nerves (Feng et al., 2011a; Mohamed and Atkinson, 1983; Rothova et al., 2011). The tooth germ starts out as an epithelial thickening (Figure 1A). This region of thickening proliferates to produce a bud that grows into the underlying mesenchyme (Figure 1B). The surrounding mesenchymal cells then condense around the bud. This region of condensing mesenchyme goes on to form the dental follicle and dental papilla of the tooth and associated periodontium, such as the alveolar bone (Diep et al., 2009). At the end of the bud stage the mesenchyme induces the

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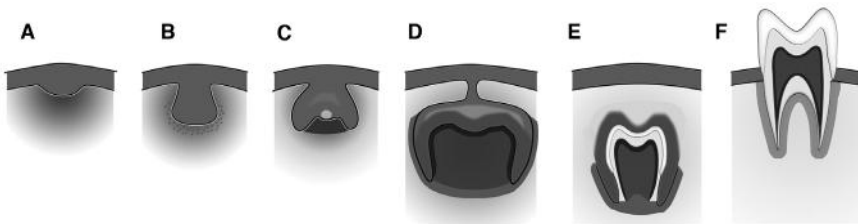
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*List of abbreviations after the text.*



**Figure 1.** Stages of mouse molar development. (A) Thickening of the oral epithelium (red). (B) Invagination of the dental epithelium to form a bud, surrounded by condensing neural crest derived mesenchyme (blue). (C) Cap stage tooth germ. Dental papilla in blue, primary enamel knot in light blue. (D) Bell stage tooth germ. The odontoblasts (dark purple) differentiate along the inner enamel epithelium, which starts to form ameloblasts. (E) Pre-eruption. Dentin (light blue) and enamel (white) form to fix the shape of the tooth. Root development is initiated. (F) Final erupted tooth. The epithelial layers of the crown are lost.

*Color image of this figure appears in the color plate section at the end of the book.*

cells at the tip of the bud to form the primary enamel knot (Jernvall et al., 1998). The primary enamel knot expresses a range of signaling molecules, transcription factors and cell cycle regulators that stimulate proliferation in the surrounding epithelium and mesenchyme, while the enamel knot itself remains non-proliferative. This difference in proliferation rate leads to a folding of the dental epithelium and the tooth germ moves from the bud to the cap stage. At the cap stage the enamel knot cells can be clearly identified as a bulge at the centre of the inner dental epithelium (Figure 1C). The enamel knot is a transient structure and disappears by apoptosis at the late cap/early bell stage (Jernvall et al., 1998). Multicuspid teeth, such as molars, go on to form secondary enamel knots, which lead to further folding of the dental epithelium, creating tooth cusps. At the bell stage the tissues of the tooth undergo cytodifferentiation, with the inner dental epithelium forming the enamel producing ameloblasts, while the adjacent mesenchymal cells in the dental papilla form dentin producing odontoblasts (Figure 1D). With the production of these hard tissues, enamel and dentin, the shape of the tooth is fixed. After formation of the crown is complete, the roots start developing, anchoring the tooth into the surrounding alveolar bone via the periodontal ligament. At eruption, the ameloblast layer is removed by mechanisms such as apoptosis, leaving a path for the eruption of the tooth, and exposing the enamel to the oral surface (Figure 1E, F).

## STEM CELLS WITHIN THE ADULT TOOTH

Stem cells have been located in various parts of the human tooth and act to replenish lost tissue. Within the dental pulp of extracted third molars (wisdom teeth) dental pulp stem cells (DPSCs) have been isolated. These



cells express some putative stem cell markers, such as STRO-1, can differentiate into odontoblasts and have been suggested to be involved in the repair of dentin (Shi et al., 2005). Stem cells have also been isolated from the pulp of human exfoliated deciduous teeth and are known as SHED cells. In contrast to DPSCs, SHED cells exhibit higher proliferation rates and can form sphere like clusters in tissue culture (Miura et al., 2003; Nakamura et al., 2009). The perivascular regions of the tooth are believed to be stem cell niches and express the stem cell marker ABCG2 (Iohara et al., 2006; Nam and Lee, 2009; Shi and Gronthos, 2003).

Periodontal ligament (PDL) stem cells have also been found within the periodontal ligament between the tooth and the alveolar bone of functional teeth, providing a source of cells that can differentiate into PDL. At the apical base of the erupting tooth SCAP stem cells have been isolated. These cells are only present during root formation, before tooth eruption, but can be isolated from extracted third molars. SCAP cells produce primary dentin of the root, compared to DPSCs that produce reparative dentin. Around extracted wisdom teeth, dental follicle stem cells (DFSC) have also been isolated, which can differentiate into cementum, bone and PDL (Morsceck et al., 2005). The SCAP and DFSC are only present in developing teeth, unlike the DPSCs and PDLSC, and are involved in the initial formation of structures, rather than their repair. These different populations of stem cells provide a valuable source of stem cells for tissue engineering (Mantesso and Sharpe, 2009; Yen and Sharpe, 2008). These stem cell populations in human lost and extracted teeth are able to form mesenchymal derivatives. There is, however, a lack of epithelial stem cells, which are necessary to provide a source of ameloblasts and produce enamel. During late tooth development and eruption the crown dental epithelium undergoes regression and apoptosis and is therefore no longer available as a source of cells. One possible remaining location of epithelial cells is the epithelial cell rests of Malassez (ERM). These cells are clumps of epithelial cells derived from Hertwig's Root sheath (HERS) and are found covering the roots of adult teeth. These cells have been isolated from periodontal ligament and can be induced to proliferate and differentiate into ameloblasts-like cells (Shinmura et al., 2008; Talic et al., 2003). Although the stem cell properties of the ERM are unclear, they represent a possible source of cells for recombination with mesenchymal dental stem cells for tooth engineering.

## **STRATEGIES OF TOOTH REPAIR/RENEWAL**

In humans teeth stop growing at eruption, with the first generation of deciduous teeth replaced by a second set of permanent teeth. This is also observed in many other mammals, such as primates, cats, dogs and pigs, and is known as diphyodonty. In some mammals, such as rodents, however,

only one set of teeth form, which is known as monophyodonty. In this case the teeth are often continuously growing, to allow for replacement of tissue at the oral surface as it is worn down. In contrast, in many non-mammalian species such as snakes and sharks, multiple generations are produced that allow an almost constant supply of new replacement teeth, known as polyphyodonty. In these species the number of tooth generation is not set. These different strategies for coping with the problems of tooth wear require different distributions and functions of stem cells. For example, it is important that diphyodont and monophyodont species have mechanisms in place for tooth repair, as once the adult teeth are gone there is no replacement and the animal's dentition is compromised. Continuously growing teeth also need a constant supply of stem cells to allow for expansion of the tooth during adult life. In contrast, repair mechanisms are less important for polyphyodont species where a replacement tooth is ready to take over the function of the previous tooth. Here it is important that stem cells are set aside for the creation of new generations. For a complete view of the role of stem cells in tooth development we therefore need to investigate their presence and function in monophyodont, diphyodont and polyphyodont species.

## **STEM CELL LOCATION IN CONTINUOUSLY GROWING TEETH**

Continuously growing teeth are observed in rodents and are associated with a single set of teeth. Both incisors and molars can be continuously growing, this being species dependent. Guinea pigs, voles and rabbits, for example, have continuously growing molars and incisors, while mice only have continuously growing incisors, reflecting the difference in the diet of these animals. Rabbit teeth have been predicted to grow 46cm a year (Shadle, 1936), with regeneration of extracted incisors possible if the dental pulp and tooth base avoid suffer serious damage (Lavagna, 1812).

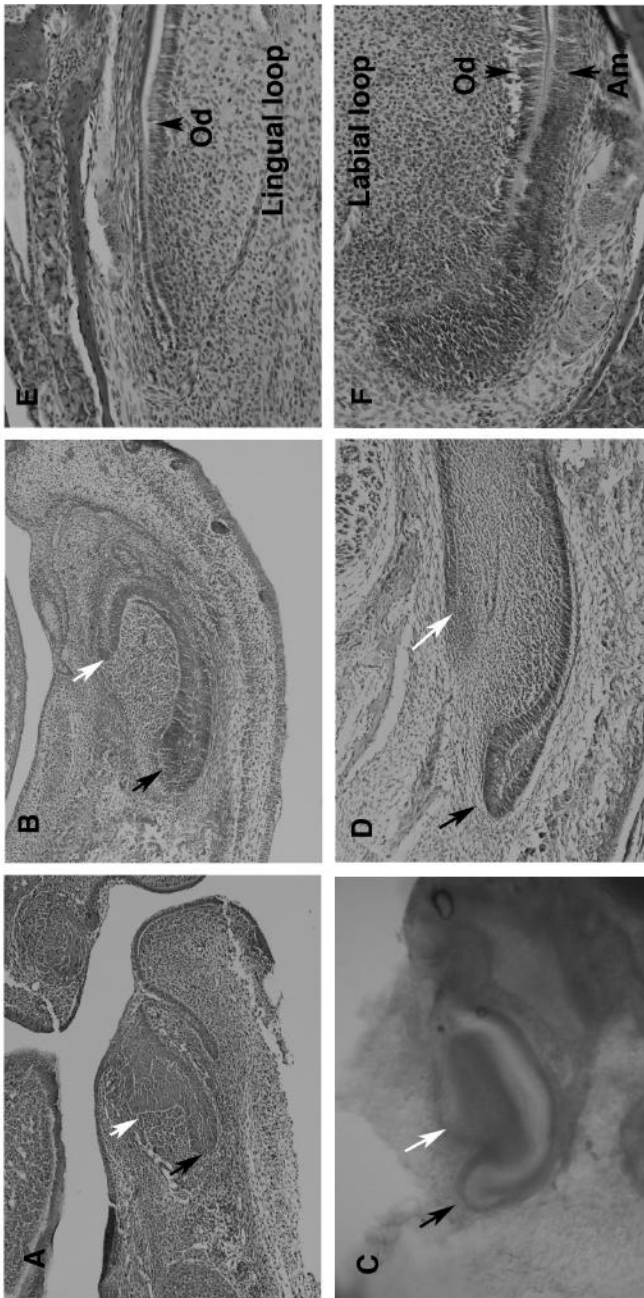
One of the most widely researched examples of continuous tooth growth is the mouse incisor. The mouse incisor is a large tooth, derived from the fusion of a number of incisor placodes during early development (Peterkova et al., 2002). For mice, that gnaw their food, the incisors are continuously worn down at the tips during their lifetime. To compensate the incisor continues to grow from its apical part. To aid tooth wear, the mouse incisor has enamel, the hardest substance in the body, only on the labial surface of the incisor to allow single face erosion. In mouse mutants with enamel on both the labial and lingual sides of the tooth the incisors cannot be worn down and grow enormous, giving the appearance of tusks (Klein et al., 2008).

In order to produce a continuously growing tooth the epithelial and mesenchymal tissue must be generated, to provide both the epithelially

derived ameloblasts and mesenchymally derived odontoblasts and pulp. Two niches of stem cells are therefore required, which must interact together to produce these two tissues at the same rate. The presence of an epithelial stem cell niche has been known and researched for the last twenty-five years, however, the more evasive mesenchymal stem cell niche is only just starting to be understood.

## THE EPITHELIAL STEM CELL NICHE

Incisors develop asymmetrically with a large labial and smaller lingual cervical loop from the late cap to bell stage onwards (Figure 2A-D). This is important as it is the epithelial cells of the labial loop that form a stem cell niche and provide a source of cells to make the ameloblasts of the continuously growing incisor. Cells within this labial loop develop into ameloblasts, stratum intermedium, stellate reticulum and outer enamel epithelium (Kawano et al., 2004). The lingual side does not form ameloblasts but functions as a root, producing epithelial cell rests of Malassez (ERM) and anchoring the tooth to the jaw (Tummers and Thesleff, 2003). The lingual epithelial loop appears not to be able to respond to signals from the surrounding mesenchyme as both lingual and labial mesenchyme can induce ameloblasts differentiation when recombined with the labial but not the lingual loop (Amar et al., 1986; Amar et al., 1989). In the adult incisor, the bulge of the labial cervical loop is clearly visible after dissection, and has been called the apical bud (Harada and Ohshima, 2004) (Figure 2E, F). Within this bud is a population of slow cycling cells, as shown by BrdU pulse-chase labeling (Harada et al., 1999). Label retention, although not a universal feature of stem cells, is shown in a number of stem cell populations and can be used as an indicator of the presence of a niche (Fuchs, 2009). DiI labeling of the central stellate reticulum cells in the labial cervical loop has indicated that cells move from the stellate reticulum out into the basal epithelium, contributing to the pool of transit-amplifying cells. These cells then move to the distal tips of the tooth, undergoing differentiation to ameloblasts as they move further away from the apical bud (Harada et al., 1999). Notch proteins are important regulators of stem cell function controlling stem cell number and cell fate decisions in a number of niches (Androutsellis-Theotokis et al., 2006). All three Notch receptors (1, 2 and 3) are expressed in the apical bud of the incisor. Notch 1 is restricted to the stellate reticulum cells at the centre of the apical bud and in the forming stellate intermedium, while Notch 3 is expressed in the whole cervical loop (Felszeghy et al., 2010b; Mitsiadis et al., 1998). Lunatic fringe, a regulator of Notch signaling, is expressed in the basal epithelium (Harada et al., 1999; Harada and Ohshima, 2004; Mitsiadis et al., 1998). Notch activation may play a role in ensuring a continuous supply of stem cell progenitors in the



**Figure 2.** Development of the cervical loops of the mouse incisor. (A, B, D, E, F) sagittal sections. (A) E14.5 cap stage tooth germ. An asymmetric development of the cervical loops is already apparent. (B) E15.5. The labial cervical loop becomes thickened in comparison to the lingual loop. (C) Dissected incisor showing the clear difference in size and shape of the loops at this stage. (D) E16.5. The Labial loop has a clear layered structure with an outer basal layer and inner stellate reticulum layer. The stem cells reside in this central region. (E) The lingual loop is very thin and no ameloblasts form adjacent to the odontoblasts (Od). (F) In contrast, the labial loop is much larger and ameloblasts (Am) differentiate adjacent to the odontoblasts. White arrows in A-D indicate lingual cervical loop. Black arrows in A-D indicate labial cervical loop.

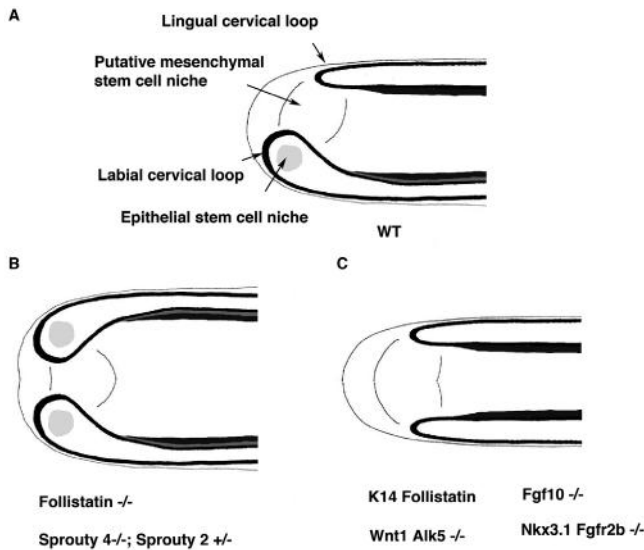
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epithelial niche. Postnatal inhibition of the Notch pathway leads to loss of *Hes1*, a Notch target gene, and a reduction in size of the cervical loop, due to reduced proliferation and a high incidence of cell death (Felszeghy et al., 2010a). As expected, this depletion of stem cells results in a reduction in the number of preameloblasts. Further evidence that the labial cervical loop is an epithelial stem cell niche comes from the expression of stem cell markers, such as Oct 3/4 (also known as Pou5f1). Oct 3/4 maintains pluripotency and self-renewal in embryonic stem cells and has been shown to be localized to the nucleus in the labial cervical loop in the continuously growing incisor after eruption (Li et al., 2011). Oct 3/4 protein is expressed in other areas of the tooth during development but shifts from the nucleus to the cytoplasm, where its function appears different (Li et al., 2011; Nakagawa et al., 2012). *Bmi-1*, a member of the polycomb gene family of transcriptional repressors, and a key regulator of stem cell self-renewal and proliferation in many tissues, is also restricted to the apical bud during postnatal development. Expression is fairly widespread during early development but becomes restricted to the labial cervical loop from E16 onwards, indicating that the stem cell niche is defined from this point onwards (Li et al., 2011). *Yap*, a transcriptional co-repressor also used as a stem cell marker, is in contrast not associated with the apical bud but with the transit-amplifying cells from E16 onwards (Li et al., 2011), indicating different roles for these stem cell markers. The stem cell marker *Lgr5*, which marks epithelial stem cells in the intestine and hair follicle, has also recently been shown to be expressed in the labial cervical loop in the stellate reticulum compartment (Suomalainen and Thesleff, 2010). The Wnt/ $\beta$ -catenin pathway has an established function in regulating stem cells in the hair follicle, skin and intestine (Haegebarth and Clevers, 2009; Lowry et al., 2005). Interestingly the epithelial stem cells niche in the cervical loops were shown to be negative for a Wnt reporter (*Axin 2*), indicating that the dental epithelial stem cells are not regulated directly by Wnt/ $\beta$ -catenin signaling (Suomalainen and Thesleff, 2010).

The genes responsible for creation of the epithelial stem cell niche during development are starting to be understood, driven by the findings of mutants where the niche is disrupted or found ectopically (Figure 3).

In follistatin mutant mice, ameloblasts-like cells form from the lingual and labial loops, indicating formation of a labial-like stem cell niche on both sides of the tooth. In follistatin-overexpressing transgenic mice, in contrast, the labial loop is smaller and does not form ameloblasts (Wang et al., 2007; Wang et al., 2004). Follistatin, therefore appears important for the inhibition of the epithelial stem cell niche, and for restricting the niche to the labial side of the forming tooth.

Follistatin is an extracellular antagonist of activin. In the absence of activin, *Bmp4* (Bone morphogenic protein 4) has been proposed to block the expression of *Fgf3* on the lingual side (Thesleff et al., 2007). This results



**Figure 3.** Signals involved in induction and maintenance of the labial cervical loop. (A) Schematic showing a WT cervical loop region of an incisor at birth. (B) The lingual cervical loop is takes on the morphology of the labial loop when follistatin or Sprouty genes are knocked out leading to a stimulation of Fgf signaling. (C) The labial cervical loop takes on the morphology of the lingual loop when Follistatin is overexpression, or if Fgf signaling is reduced.

in Fgf3 being expressed only on the labial side of the developing tooth, and, along with Fgf10, maintains ameloblast precursor cells on this side of the tooth (Harada et al., 1999; Harada et al., 2002). In Fgf10 knockout mice the labial cervical loop and stem cell compartment does not form correctly due to reduced proliferation in the epithelium and the resultant tooth is of reduced length (Harada et al., 2002). Fgf3 knockout mice incisors appear normal at birth, but compound Fgf3  $-/-$ ; Fgf10  $+/-$  mice have a severely hypoplastic labial cervical loop, indicating that precise levels of Fgf signaling are important for regulating the size and position of the stem cell niche (Wang et al., 2007). The labial loop is also disrupted when Fgfr2b, the main receptor for Fgf10 and Fgf3, is conditionally knocked out in the dental epithelium (Lin et al., 2009). Fgf signaling is also needed after birth, with postnatal attenuation of signaling through the Fgfr2b receptor leading to a loss of proliferation in the transit-amplifying cells, and a failure of enamel formation (Parsa et al., 2010).

Tgfb signaling also appears to act upstream of Fgfs to control formation of the niche, as loss of the receptor Alk-5 in the dental mesenchyme leads to a reduction in size of the labial cervical loop and loss of Fgf expression (Zhao et al., 2011). In keeping with the phenotypes generated from loss of Fgf signaling, when Fgf signaling is enhanced, by knockout of the Fgf inhibitors Sprouty 2 and 4, an epithelial stem cell niche is created on both

sides of the incisor and ameloblasts differentiate on both sides (Figure 3). Recently a number of microRNAs have been shown to be expressed in the labial cervical loop, indicating another level of complexity of regulation of this population (Jheon et al., 2011).

An epithelial stem cell niche has also been observed in continuously growing vole and guinea pig molars. Similar to the mouse incisor, epithelial bulges at the base of the vole molar are maintained by mesenchymal Fgf10 expression, and express Notch, in contrast to the lack of expression of these key genes in the adult mouse molar, which does not continuously grow (Tummers and Thesleff, 2003). The maintenance of Fgf10 expression therefore appears to be a key player in the move to continuous tooth growth. In the guinea pig, slow-cycling label retaining cells were located in four regions of the cervical loops of the molar at the base of the tooth (Hashimoto et al., 2008). Persistent cervical loops have also been described in sloth molars, which are continuously growing (Tummers and Thesleff, 2008).

## **THE MOUSE INCISOR MESENCHYMAL STEM CELL NICHE(S)**

### **Growth**

Since the mouse incisors grow continuously throughout life to compensate for loss of tissue from their tips, both odontoblasts and ameloblasts need to be continuously added to the base (cervical end) of the tooth to maintain growth. Unlike enamel, the formation of dentin is not restricted to one side of the tooth, thus odontoblasts need to be continuously formed adjacent to the epithelium on the lingual and labial sides of the tooth. For mesenchymal cells that form the pulp and odontoblasts this is achieved by a stem cell niche that appears to be located between the two cervical loop areas (Figure 3). A small area of very slow cycling cells detected at the most cervical end of the incisor is indicative of possible mesenchymal stem cells (Seidel et al., 2010). Cells in this area express the Shh target gene, Gli1. When a Gli1-ERTCre was used to genetically label these cells, it was shown that their progeny could be found in the pulp and odontoblast cell layer (Seidel et al., 2010). This suggests that Shh-responsive cells in the area of slow cycling mesenchyme cells do contribute to the formation of new pulp cells and odontoblasts during incisor growth. Shh-responsive cells are also observed in the epithelial cervical loops, indicating that this one signaling pathway might be involved in coordinating the production of epithelial and mesenchymal tissue.

If these mesenchymal stem cells are very slow cycling then there must be a population of highly proliferative cells, equivalent to transit-amplifying cells that provide sufficient cells to differentiate into the mesenchymal cell

types of the growing tooth. High proliferating cells can be visualized more distally to the slow cycling cells although there is as yet no evidence that these are transit-amplifying cells.

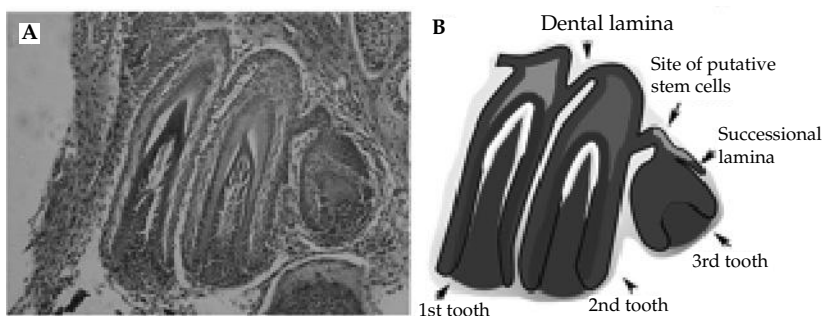
## **Repair**

When teeth are damaged such that the pulp is exposed, a natural repair process is initiated to prevent both infection and blood loss. New odontoblasts differentiate and produce dentine to repair the damage. In the mouse, repair of equivalent damage to molars (non-growing) and incisors (growing) stimulates a more rapid dentine repair in incisors. This suggests that the mesenchymal stem cells involved in growth may have a role in repair. DiI-labelling of cells in the region of the pulp containing the putative transit amplifying cells in damaged teeth revealed directed migration (homing) of labeled cells towards the site of damage that was accompanied by odontoblast differentiation (Feng et al., 2011b). This was indicative of tooth damage mobilizing mesenchymal cells to elicit a repair. The kinetics of migration and differentiation however suggested that this response was not the only source of new odontoblast cells. By using the ERT-cre system to label perivascular cells (pericytes) that express NG2, proliferation and differentiation of pericytes into odontoblasts at the site of damage indicated that mesenchymal stem cells derived from pericytes were also mobilised in response to damage (Feng et al., 2010). The stem cell marker ABCG2 was found expressed in the incisor mesenchyme during development, particularly associated with the perivascular region (Li et al., 2011). Thus it appears that at least two separate sources of stem cells in the incisor are able to respond to damage. The lack of a non-pericyte response in molars may explain why repair takes longer than in incisors.

## **STEM CELLS FOR REPLACEMENT DENTITIONS**

In order to understand where the stem cells niche lies in animals that continually replace their teeth, researchers have moved away from mammals to study fish and reptiles. In fish (chondrichthyns and some teleosts) and reptiles, the replacement dentition forms from a successional lamina, closely associated with the previous tooth (Buchtova et al., 2008; Huyseune, 2006; Huyseune and Thesleff, 2004; Smith et al., 2009) (Figure 4A). In diphyodont mammals, such as the ferret and pig, the permanent tooth also forms from a successional lamina (Jarvinen et al., 2009; Stembirek et al., 2010). In such mammals, this lamina disintegrates after initiation of the permanent tooth preventing the formation of any further tooth germs (Buchtova et al., 2012; Stembirek et al., 2010). In monophyodont mammals





**Figure 4.** Snake replacement tooth development. (A) Trichrome stained section through a corn snake mandible showing formation of a series of teeth from the dental lamina. (B) Schematic highlighting the location of the putative stem cells (green) on the lingual side of the dental lamina, behind the successional lamina at the tip of this structure.

*Color image of this figure appears in the color plate section at the end of the book.*

no successional lamina appears to form, while in monophyodont reptiles this lamina is present but then regresses (Richman and Handrigan, 2011). In polyphyodont reptiles, however, the dental lamina and successional lamina are permanent structures. In chondrichthyans stem cells have been predicted to reside in the lamina (Smith et al., 2009). The role of stem cells in creation of the replacement dentition has been studied in the leopard gecko, *Eublepharis macularius* (Handrigan et al., 2010). Pulse-chase BrdU experiments have shown the location of label-retaining cells on the lingual side of the dental lamina, close to the successional lamina (Figure 4B). A subset of these label-retaining cells express stem cell markers, such as *Lgr5*, *Dkk3* and *Igfbp5* (Handrigan et al., 2010). *Lgr5*, *Dkk3* and *Igfbp5* are characteristic of stem cells in the mammalian hair bulge, while *Lgr5* has also been shown to be expressed in the mouse labial cervical loop of the incisor (Suomalainen and Thesleff, 2010). Presence of these markers indicates that these are indeed putative stem cells. In mammalian intestine and hair follicles an increase in canonical Wnt signaling induces quiescent stem cells to form transit-amplifying cells (Blanpain et al., 2004; Haegebarth and Clevers, 2009; Lowry et al., 2005). Wnt signalling also appears to regulate the putative stem cells in the gecko dental lamina, with stimulation of the canonical Wnt pathway leading to an increase in proliferation in the lingual region (Handrigan et al., 2010). In teleosts, such as zebrafish and cichlids, the initial teeth do not form from a dental lamina but from the epithelium at the oral/pharyngeal surface. In zebrafish the replacement teeth develop at the base of the epithelial crypts that surround the erupted, functional teeth, indicating that this crypt may be a niche for dental stem

cells. [3H]Thymidine incorporation to follow proliferation in cichlids has also indicated that such niches might contain a stem cell population (Huyseune, 2006). In teleosts, the eruption of the previous tooth may act as a stimulus for induction of the replacement tooth from this stem cell population. In the salmon the middle dental epithelium may substitute for a successional lamina, and has been hypothesized to be the source of stem cells (Huyseune and Witten, 2008). More information, however, is necessary before any more definite conclusions can be made about the localization of stem cells during tooth regeneration, and in particular analysis of putative stem cell markers and label retaining cells in fish may shed light on the location of a stem cell niche.

## **CONCLUSIONS**

Stem cells play an important role in tooth development, repairing damaged teeth, allowing continuous growth, and providing a source of cells for development of replacement teeth. In many areas we are just starting to understand where the stem cell niches are located, and the signals involved in their creation and maintenance. In general current research has focused on the differentiation of a specific tissue during repair/development. Few studies have investigated stem cells function and localization in the placode/oral epithelium and mesenchyme, which have the potential to form an entire organ. More information is also needed to understand how different stem cell niches are coordinated, for example, allowing epithelial and mesenchymal tissue to be created at a uniform rate in a continuously growing tooth. Whether the dental stem cell niches have a bi-compartmentalised structure, as previously shown for hair, intestine and haematopoietic stem cells would be interesting to investigate (Greco and Guo, 2010). With the advent of new genetic technology, we are beginning to understand stem cells in the mouse tooth, while a renewed interest in non-standard animal models has paved the way for a better understanding of the role of stem cells in replacement dentitions.

## **ACKNOWLEDGEMENTS**

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## ABBREVIATIONS

|      |                                                              |
|------|--------------------------------------------------------------|
| SHED | Stem cells from the pulp of human exfoliated deciduous teeth |
| DPSC | dental pulp stem cells                                       |
| PDL  | Periodontal ligament                                         |
| DFSC | dental follicle stem cells                                   |
| ERM  | epithelial cell rests of Malassez                            |
| HERS | Hertwig's Root sheath                                        |
| SCAP | stem cells from apical papilla                               |

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# Liver and Pancreas: Mechanisms of Development and Size Control

*Ravi Maddipati and Ben Z. Stanger\**

## SUMMARY

The Gastrointestinal (GI) system is primarily comprised of the liver, pancreas and luminal tract. Together, these organs play a critical role in nutrient homeostasis, synthetic function and toxin removal (Table 1). The liver is mainly responsible for sustaining serum protein and metabolite concentrations, drug metabolism, detoxification and bile production. The endocrine pancreas works to tightly control serum glucose metabolism while its exocrine portion aides in digestion of nutrients. The GI tract functions to digest food, extract nutrients and dispose of waste. Despite their varied functions, these organs share a common embryological origin, being derived primarily from the endoderm with contributions from mesoderm and ectoderm germ layers (Table 2). Advances over the past 1–2 decades have given us insight into the genes, transcription factors and pathways involved in the patterning, morphogenesis and differentiation of gut endoderm.

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*List of abbreviations after the text.*

**Table 1.** physiologic functions of the gastrointestinal system.

| <b>Gastrointestinal components and function</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |  |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|
| <b>Hepatobiliary (Liver, Gallbladder, Bile ducts)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |  |
| <ul style="list-style-type: none"> <li>- toxin clearance</li> <li>- protein, coagulation factors, bile acid production</li> <li>- lipid/cholesterol synthesis and glucose metabolism</li> <li>- nutrient homeostasis</li> </ul>                                                                                                                                                                                                                                                                                                                      |  |
| <b>Pancreas</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |  |
| <ul style="list-style-type: none"> <li>- Exocrine: breakdown of proteins, complex carbohydrates and lipids</li> <li>- Endocrine <ul style="list-style-type: none"> <li>▪ <math>\alpha</math> cell: glucagon → glucose uptake</li> <li>▪ <math>\beta</math> cell: insulin → glucose uptake</li> <li>▪ <math>\delta</math> cell: somatostatin → release of other GI hormones</li> <li>▪ PP cell: insulin → regulation of pancreatic secretory function</li> <li>▪ <math>\epsilon</math> cell: insulin → induces hunger response</li> </ul> </li> </ul> |  |
| <b>Intestines (esophagus, stomach, small &amp; large intestines)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |  |
| <ul style="list-style-type: none"> <li>- digestion and absorption of nutrients</li> <li>- water reabsorption</li> <li>- toxin removal</li> </ul>                                                                                                                                                                                                                                                                                                                                                                                                     |  |

**Table 2.** origin of tissues from the germ layers.

| <b>Germ layer derivatives</b>                                                                                                                                                                           |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Ectoderm</b>                                                                                                                                                                                         |
| <ul style="list-style-type: none"> <li>- nervous system</li> <li>- epidermis and epithelial linings</li> <li>- pituitary glands</li> </ul>                                                              |
| <b>Mesoderm</b>                                                                                                                                                                                         |
| <ul style="list-style-type: none"> <li>- heart, blood, vessels, lymphatics, spleen</li> <li>- muscle and bone</li> </ul>                                                                                |
| <b>Endoderm</b>                                                                                                                                                                                         |
| <ul style="list-style-type: none"> <li>- liver, gallbladder, bile ducts, Pancreas, gut tube (esophagus, stomach, intestines)</li> <li>- lungs</li> <li>- thyroid, thymus, parathyroid glands</li> </ul> |

This progress has allowed us to better understand the mechanisms that drive organogenesis, the role of stem cell and fetal progenitors in tissue growth and the methods by which organs regulate their size. This chapter reviews our current understanding of liver and pancreas development with a particular emphasis on the role of stem/progenitor cells in these tissues and mechanisms of organ size determination and growth.



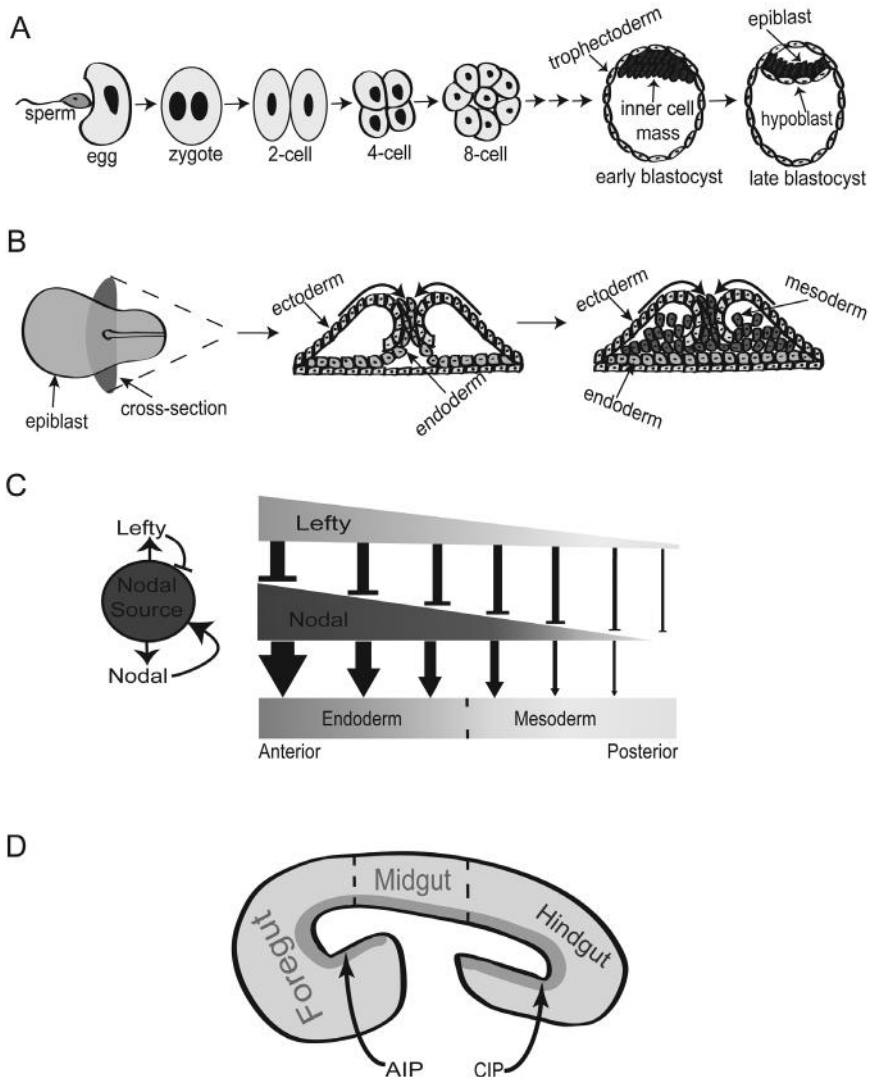
## INTRODUCTION

### Endoderm Development

Following egg fertilization and cleavage, blastulation occurs and the resulting blastocysts are implanted in the uterus (at embryonic day 4.5 in the mouse, or E4.5) (Figure 1a). Subsequent formation of the node and primitive streak in the posterior half of the epiblast marks the beginning of germ layer formation (gastrulation) and ensuing migration of epiblast cells through the primitive streak gives rise to the endoderm, mesoderm and ectoderm (Figure 1b). The endodermal portion of this trilaminar embryo subsequently undergoes a series of folding steps resulting in formation of a primitive gut tube. Patterning of this tubular structure ensues and establishes the broad endodermal domains known as foregut, midgut and hindgut. Through continued interactions with underlying mesoderm these regions are progressively specified into smaller groups of tissue-specific progenitor pools. Continued expansion of these progenitor populations gives rise to individual organ buds (morphogenesis) which eventually differentiate into distinctive cell types and tissue structures. The foregut region gives rise to the lungs, trachea, thyroid, esophagus, stomach, liver, pancreas and biliary system, while the midgut and hindgut develop into the small and large intestine respectively (Figure 1d). The coordination of these sequential steps occurs under the control of genetic and transcriptional regulatory mechanisms that operate in a temporal and context dependent manner, thus allowing them to serve many different functions throughout development.

### Endoderm Specification and Gut Tube Formation

Initial formation of the definitive endoderm begins with the movement of epiblast cells into and through the primitive streak. Early studies documenting the fates of cells in the epiblast (fate maps) suggested that precursors of the endoderm arise from a region adjacent to the anterior extension of the primitive streak (Lawson et al., 1991). These fate maps also outline areas of overlap between endoderm- and mesoderm-forming regions in the midstreak epiblast. Whether this indicates a bipotential mesendodermal progenitor as the source for these germ layers is still a matter of controversy. Although *in vivo* mammalian data are inconclusive, studies in lower invertebrates and *in vitro* embryonic stem cell differentiation systems suggest that such precursors exist (reviewed in Lewis and Tam, 2006). The formation of endoderm and mesoderm from mesendodermal progenitors is primarily driven thru activation of germ layer specific factors by the TGF $\beta$  family growth factor Nodal (Feldman et al., 1998; Shen, 2007).



**Figure 1. overview of endoderm development.** (A) Progression from fertilized egg to blastocyst. (B) Cross-sectional view of the epiblast demonstrating migration of the three germ layers. Ectoderm (pink), Mesoderm (red), endoderm (blue). (C) Effects of Lefty and nodal on specification of endoderm and mesoderm in the epiblast. High nodal signaling overcomes left inhibition and specifies endoderm while further from the nodal source lefty inhibition of nodal has greater effect and leads to mesoderm development. (D) The segregation of the endoderm in the foregut, midgut and hindgut domains along with locations of the Anterior intestinal portal (AIP) and Caudal intestinal portal (CIP).

*Color image of this figure appears in the color plate section at the end of the book.*

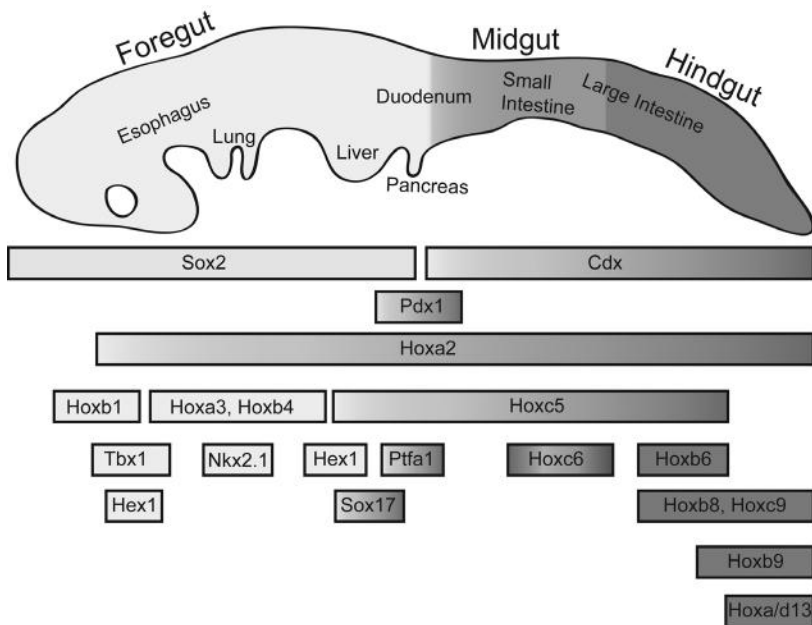
Differential expression of these genes occurs in a dose-dependent manner such that endoderm and mesoderm arise from areas of high and low nodal concentration respectively (Green and Smith, 1990; Shen, 2007) (Figure 1c).

Following endoderm formation, establishment of the primitive gut tube takes place. Initiation of this process occurs under the guidance of various endodermal genes independent of mesoderm or ectoderm input. Initial folding of the anterior endoderm results in formation of the Anterior Intestinal Portal (AIP), a blind loop structure that gives rise to foregut and ventral structures. This is followed by a similar process in the posterior region resulting in the Caudal Intestinal Portal (CIP), site of the future hindgut (Figure 1d). Subsequent steps result in inward migration of the AIP toward the yolk stalk and eventual lumen formation via ion channel-mediated fluid accumulation (Bagnat, 2007). Alterations in the signaling mechanisms underlying this process can have dramatic effects. For example, disruption of folding by mutations in *Hnf1 $\beta$*  or the *Tgf- $\beta$*  family member *furin* can lead to an absence of foregut and midgut structures (Ang and Rossant, 1994; Roebroek et al., 1998). Thus it appears that proper formation of the gut tube is critical for initiation of endoderm organ development within the body.

## **Mechanisms of Endodermal Patterning**

Concurrent with germ layer development during gastrulation, patterning (establishment of distinct organ domains) within the endoderm begins to take shape. Initially, endoderm patterning is subject to the influences of the nodal pathway, resulting in the correct positioning of tissues along the anterior-posterior (A-P) axis of the embryo. Similar to its role in germ layer specification, nodal signaling establishes A-P endoderm fates through a classical morphogen gradient (Shen, 2007). Specifically, anterior endoderm fates (e.g., lung, liver, esophagus) are promoted by high levels of nodal and low levels of its inhibitor, *lefty*, whereas low levels of nodal and high levels of *lefty* lead to posterior endoderm formation (Lowe et al., 2001; Liu et al., 2004) (Figure 1c). The ability of this nodal gradient to direct A-P patterning is regulated primarily through expression of the transcription factors *Mixl1*, *Foxa2* and *Sox17*. Genetic models in which these factors are selectively removed demonstrated that high nodal levels induce anterior fates through expression of *Mixl1* and *Foxa2* whereas low levels conferred a posterior fate through *Sox17* production (Dufort, 1998; Hart et al., 2002; Kanai-Azuma et al., 2002; Lewis, 2006).

In addition to the effects of nodal, endoderm patterning is guided by spatially-defined expression patterns of homeobox-containing genes within the underlying mesenchyme (Figure 2). These DNA binding transcriptional regulators are comprised of four Hox clusters (a-d), a set of “dispersed” homeobox genes (Nk2.1, Hlx, Hex) and “para-hox” genes (Pdx1, Cdx2, Gsx1), categories based on the position of genes within the genome (Beck et al., 2000). The precise positioning of Hox genes within the chromosome seems to be particularly important for anterior-posterior patterning as expression of hox genes spreads along the A-P axis in a manner that directly reflects their underlying chromosomal order (Deschamps et al., 1999). Such “co-linear” expression (resulting in a “Hox code”) allows for coordination of positional identities along the A-P axis in all three germ layers and expression of Hox genes in the mesoderm and ectoderm often results in re-ordering of axial or neural tissue to reflect the new Hox gene arrangement in what is termed a “homeotic” transformation (Beck et al., 2002). Such changes in fate are also observed upon loss or mis-expression of para-hox genes in the endoderm, suggesting a role for these factors in



**Figure 2. patterns of hox, dispersed homeobox and para-hox genes along the gut tube.** The upper panel depicts the gut tube with foregut, midgut and hindgut regions and associated organ buds along the A-P axis of the gut tube. The bottom panel highlights important homeobox transcription factor expression patterns along the gut tube. The expression domains of these factors often overlap and can change in a temporal fashion throughout development. This figure was adapted from Zorn and Wells, 2009.

establishing organ boundaries. For example, conditional ablation of the hindgut para-hox gene *Cdx2* leads to replacement of posterior intestinal epithelium with squamous epithelium, reflecting an extension of foregut/esophageal expression domains into the hindgut region. Conversely, ectopic expression of *Cdx2* in anterior domains leads to extension of hindgut intestinal epithelium into the foregut region (Gao et al., 2009). Thus organs domains in the early gut endoderm are at least in part defined by the collection of homeobox-containing genes within the endoderm and by a gradient of nodal signaling.

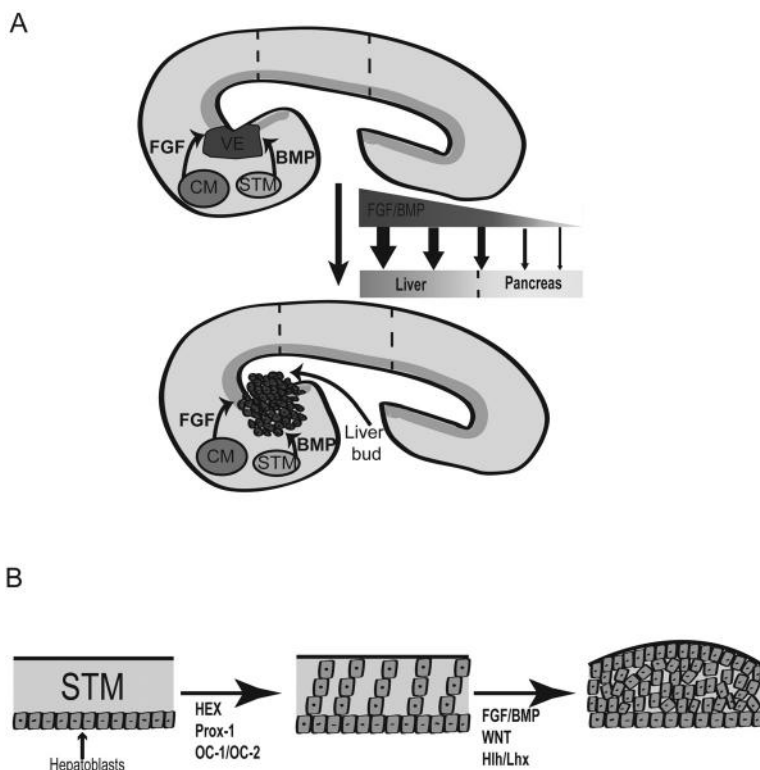
## **ORGAN SPECIFICATION AND MORPHOGENESIS**

Following the initial regionalization of early endoderm, these broad domains (foregut, midgut, hindgut) are further specified to become defined organ domains. Much of this specification occurs through the exchange of information between the endoderm and underlying mesodermal structures (Wells and Melton, 2000) and the mediators of this process consist mainly of members of the Fgf, Bmp, Wnt/ $\beta$ -catenin and Retinoic acid families of proteins. These signals function in either an instructive manner, by which cells are directed in their developmental course, or a permissive manner, by which cells are allowed to progress to pre-assigned fate. These instructive and permissive signals eventually result in endoderm regions that are capable of undergoing organ specific morphogenesis and differentiation. Such signals are used throughout liver and pancreas development and have different effects depending on the embryonic stage (temporal) or position (spatial) in which they are present.

### **Liver Specification**

The initial signs of hepatogenesis become apparent soon after formation of the early definitive endoderm. Albumin expression is one of the earliest markers of hepatic induction and analysis of the transcriptional elements responsible for albumin activation more than 15 years ago revealed a role for FoxA and Gata4 transcription factors in establishing early hepatic competence (Gualdi et al., 1996; Bossard and Zaret, 1998). Through their ability to bind and activate the albumin enhancer, these factors appear to prime cells within the region of endoderm fated to become liver, facilitating further steps involved in liver development (Cirillo et al., 1998, 2002; Cirillo and Zaret, 1999). Defects in this transcriptional regulation, as seen in FoxA1/2 knockout mice, result in a lack of response to further developmental cues and subsequent failure to initiate liver formation (Lee et al., 2005).

Subsequently, a liver progenitor cell population begins to arise in competent foregut endoderm under the guidance of mesoderm-derived signals. The importance of this meso-endodermal relationship was initially revealed by transplant studies in which foregut endoderm failed to express hepatic markers in the absence of adjacent cardiac mesoderm (Le Douarin, 1975; Gualdi et al., 1996). The molecular mediators of this relationship were later found to be members of the Fgf family of signaling proteins (Jung et al., 1999; Calmont et al., 2006) (Figure 3a). Cultures of isolated foregut endoderm revealed that blocking of Fgf signaling inhibited further hepatic specification whereas addition of Fgf1 and Fgf2 was sufficient to replace cardiac mesoderm and induce albumin expression (Jung et al., 1999). Similar explant studies revealed that in addition to Fgf signals, Bmps derived



**Figure 3. liver specification and morphogenesis.** (A) Influence of Fibroblast Growth Factor (FGF) and Bone Morphogenetic Protein (BMP) secreted by the Cardiac Mesoderm (CM, red) and Septum Transversum Mesenchyme (STM, blue) respectively, on the specification of Ventral Endoderm (VE) into liver and pancreas with formation of the nascent liver bud and hepatoblasts (dark green cells) in regions of high FGF and BMP signaling. (B) Migration of the hepatoblasts (green cells) into the STM (pink) to form the liver bud.

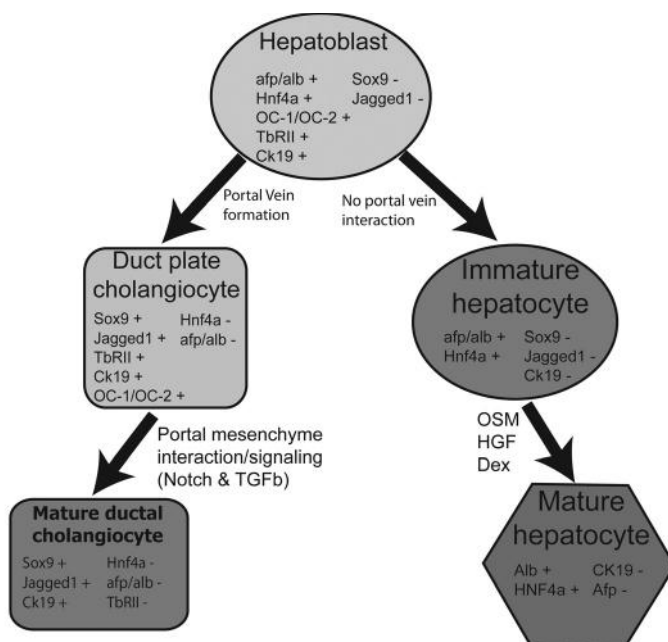
*Color image of this figure appears in the color plate section at the end of the book.*

from underlying septum transversum mesenchyme (STM) were necessary for later steps in hepatic specification and morphogenesis (Rossi et al., 2001) (Figure 3a). The ability of both signals to coordinate proper hepatic development is likely related to precise temporal and spatial crosstalk. For example, one recent study has demonstrated that Tgfb $\beta$  inhibits the effects of Fgf/Bmp until the endoderm is positioned in the correct inductive environment (Wandzioch and Zaret, 2009).

## **Liver Morphogenesis and Growth**

Liver budding and outgrowth begin soon after hepatic fate has been established. As the cardiac mesoderm moves anteriorly, hepatic progenitors (hepatoblasts) in the foregut endoderm begin to migrate and expand into the STM, forming the early hepatic diverticulum. As these early hepatoblasts continue to move towards the STM, they begin to undergo a change in their cellular morphology from a cuboidal to a pseudostratified columnar epithelium (Bort et al., 2006; Shiojiri and Sugiyama, 2004). This change facilitates degradation of the laminin-rich basal surface of the hepatic diverticulum and allows the hepatoblasts to successfully invade into the mesenchyme (Figure 3b). Initial control of this migratory process involves activation of the homeobox-containing genes *Hhex*, *Prox1* and *Onecut1/2*. Following specification, *Hhex* (which is initially expressed throughout the ventral endoderm) becomes enriched in the hepatic regions and failure to initiate *Hhex* expression during morphogenesis arrests hepatoblasts in a cuboidal state and prevents further delamination into the STM (Bort et al., 2004, 2006). Shortly after *Hhex* activation, *Prox1*, *Onecut 1* (*Oc-1* or *Hnf6*) and *Onecut-2* (*Oc-2*) are expressed and participate in subsequent stages of the delamination process through repression of E-cadherin and up-regulation of metalloproteinase at the basal surface (Sosa-Pineda et al., 2000; Margagliotti et al., 2007) (Figure 4).

After hepatoblast invasion into the septum transversum, the liver bud undergoes a period of rapid growth and proliferation (E9.5 to E15.5 in mouse) that is driven by many of the same factors employed during early hepatic development. Mesenchyme-derived Fgfs, Bmps and Wnts are some of the essential factors that regulate bud expansion, as defects in these factors is associated with delayed bud growth and/or decreased hepatoblast proliferation, resulting in a small liver size at birth (Jung et al., 1999; Rossi et al., 2001; Calmont et al., 2006; Berg et al., 2007; McLin et al., 2007). Similar effects are seen following mutation of the homeobox genes *Hlx/Lhx2* and/or *Hgf* (hepatocyte growth factor) (Hentsch et al., 1996; Schmidt et al., 1995). While *Hlx* seems to produce its effects through control of a paracrine factor, defects in *Hgfs* ability to bind the c-met receptor on hepatoblasts leads to significant apoptosis and subsequent liver hypoplasia and anemia.



**Figure 4. differentiation of hepatoblasts into hepatocytes and cholangiocytes.** Hepatoblasts adjacent to the portal vein form duct plate cholangiocytes and mature into fully differentiated biliary cells through the effects of Notch and TGF $\beta$  from the portal mesenchyme. Lack of interaction with portal mesenchyme leads to a default fate of hepatocyte formation.

This critical role for hepatocyte apoptosis in regulating embryonic liver growth is also revealed by studies of c-Myc, c-Jun, Sek1, Tnf/Nf- $\kappa$ B and Xbp (reviewed in Zorn, 2008).

## Hepatic Differentiation

With the continued growth of the nascent liver bud, hepatoblasts in the rapidly expanding region manifest their bi-potential nature as they differentiate into hepatocytes and biliary epithelial cells (BECs) (Figure 4). This process becomes apparent around E13 of mouse development as hepatoblasts in contact with the portal vein form a layer of cells called the ductal plate and express markers of biliary precursors such as cyokeratin-19 (Ck19) and Sox9. Starting at around E16, through a process known as duct plate remodeling, bile ducts form through a two-step process of differentiation (described below). In the absence of signaling from surrounding portal mesenchyme to maintain expression of BEC transcription factors, the remaining hepatoblasts not in contact with the portal vein differentiate into hepatocytes (reviewed in Zorn, 2008) (Figure 4).



The continued maturation of hepatocytes is dependent on various growth factors such as Hgf, corticosteroids and Oncostatin M (Osm) (Kamiya et al., 2001; Michalopoulos et al., 2003; Schmidt et al., 1995). As in morphogenesis, HGF is also crucial for proliferation of hepatoblasts, such that its absence leads to small liver size and significant parenchymal cell loss (Schmidt et al., 1995). Alternatively, in organoid tissue culture models, dexamethasone can suppress this growth and is critical for potentiating the hepatocyte maturation process (Michalopoulos et al., 2003). Similarly, Osm, which is secreted by hematopoietic cells residing in the fetal liver, also promotes hepatocyte differentiation through its activation of Gp130 and Jak/Stat3 signaling pathways (Kamiya et al., 1999; Matsui et al., 2002). These growth factors regulate hepatocyte maturation through modulation of a complex inter-regulatory network of transcription factor consisting of Hnf4 $\alpha$ , Hnf1 $\alpha$ / $\beta$ , FoxA2 and C/EpP $\beta$  (Si-Tayeb et al., 2010). These factors work to repress expression of BEC genes thus favoring hepatocyte development. Hnf4 $\alpha$ , a factor reported to occupy nearly 12% of genes in human hepatocytes (Odom et al., 2004), plays a central role in coordinating this network (Kyrnizi et al., 2006) as evidenced by an inability of hepatocytes to develop normal morphology, polarity and express mature liver enzymes in its absence (Battle et al., 2006; Parviz et al., 2003; Li et al., 2000).

As hepatocytes mature, zonation of liver parenchyma takes place through the expression of transcriptional factor gradients that are dependent on hepatocyte location in relation to portal triads and central veins. Zone 3 (peri-venous) hepatocytes, under the influence of a local Wnt gradient, activate the transcription factor Lef1 and induce expression of glutamine synthetase and cytochrome p450. This zone 3 phenotype is counteracted in zone 1 (peri-portal) hepatocytes (which are not exposed to Wnt) by expression of the Apc complex and Hnf4 $\alpha$ , leading to up-regulation of genes such as glutaminase-2 which are characteristic of zone 1 hepatocytes (Kaestner, 2009; Benhamouche et al., 2006; Colletti et al., 2009). This zonation of liver parenchyma is important as it allows hepatocytes near the portal and central veins to cope with different metabolic environments.

## **Biliary Differentiation**

In concert with hepatocyte development, hepatoblasts also give rise to cholangiocytes (part of BECs) in the ductal structures of the biliary tree. The biliary system is composed of intra and extra hepatic ductal systems, which function to drain bile from hepatocytes and deliver it to the lumen of the duodenum. Although connected during adulthood, these two separate ductal systems have distinct embryological origins. The intra-hepatic biliary ducts (IHBDs) arise from hepatoblasts within the liver bud (Van Eyken et al., 1998) whereas the extra-hepatic biliary ducts (EHBDs) arise from a pool of

pancreato-biliary progenitor cells in the foregut endoderm near the ventral pancreas. The hepatic duct, which serves as link between these two systems, derives its intra-hepatic portion from the liver bud and its extra-hepatic region from the hepatic diverticulum (Tan and Moscoso, 1994).

Evidence of IHBD formation is first detected around E14 in the mouse with the appearance of a monolayer of biliary pre-cursors that surround individual portal veins. These “ductal plates” subsequently develop luminal structures (E15–E16) and exhibit a transient asymmetry with the luminal side adjacent to the portal vein being lined by biliary precursor cells and the parenchymal side of the lumina consisting of hepatoblast-like cells. As these lumens become surrounded by periportal mesenchyme, this asymmetry disappears and the hepatoblast-like cells differentiate into cholangiocytes (Antoniou et al., 2009; Zong and Stanger, 2011).

IHBD development is dependent on local cues derived from adjacent portal mesenchyme and modulation of these signals by various transcription factors. A prime example of this is the Tgfb family, which is crucial for biliary differentiation. Tgfb2 and Tgfb3 are expressed primarily in the portal mesenchyme and generate a SMAD-mediated signal that weakens as it progresses into the liver parenchyma. This porto-mesenchymal gradient of Tgfb signaling suppresses hepatocyte gene expression and allows for BEC development in a specially (peri-portal) restricted manner. Studies in which Tgfb is inhibited result in impaired biliary differentiation, whereas addition to cultured hepatoblasts leads to expression of biliary markers (Clotman et al., 2005). This Tgfb signaling gradient is likely shaped by the transcription factors Oc-1 (HNF6) and Oc-2. In Oc-1 knockout mice periportal hepatoblasts exhibit mixed hepatocyte and biliary cell features and upon deletion of both Oc-1 and Oc-2 all hepatic cells exhibited this mixed phenotype. These findings are consistent with a model by which Oc-1 and Oc-2 shape a gradient of Tgfb responsiveness, limiting it to the peri-portal region (Clotman et al., 2005; Lemaigre, 2010).

Additional cues from the portal mesenchyme were discovered through studies of Alagille syndrome, a disorder in which mutations of Notch2 or the Notch ligand, Jagged1 lead to a paucity of bile ducts (McDaniell et al., 2006; Li et al., 1997). Zebrafish studies demonstrated that Notch activation leads to differentiation of hepatoblasts into biliary cells whereas its inhibition leads to a reduction in ductal plate cells (Tchorz et al., 2009). Further studies in mice also showed defects in tubulogenesis with inactivation of Hes1 (a notch target) or liver-specific deletion of Notch2 (Geisler et al., 2008; Kodama et al., 2004). Much like Tgfb, Notch may restrict ductal development to the periportal region since expression of Jagged1 is limited to portal mesenchyme and biliary cells and Notch signaling requires direct cell-cell contact (Zong et al., 2009). Studies of Wnt, Fgf and Bmp also demonstrate a role for these signaling molecules in biliary differentiation, although it

is unclear if they also provide spatial cues (Hussain et al., 2004; Yanai et al., 2008).

In contrast to the hepatoblast-derived intrahepatic ducts, the extrahepatic system is derived from cells located in the same region of endoderm that is fated to become ventral pancreas. Indeed, lineage tracing experiments suggest that EHBDs are derived from a common pancreatobiliary progenitor pool characterized by co-expression of the pancreatic marker Pdx1 and the EHBD marker Sox17. These studies further indicate that Sox17 is critical for EHBD development, as Sox17 deletion leads to ectopic pancreatic tissue while Sox17 mis-expression leads to ectopic EHBDs (Spence et al., 2009). Transcriptional regulation of EHBD differentiation is heavily dependent on the Hhex transcription factor, as evidenced by the replacement of ducts by duodenal-like tissue in its absence (Hunter et al., 2007). Additional events are also mediated by Hnf6, Hnf1 $\beta$  and Hes1, which play important roles during morphogenesis (Clotman et al., 2002; Coffinier et al., 2002; Spence et al., 2009).

## **Pancreatic Specification**

Pancreatic development begins with specification of the ventral and dorsal foregut in two distinct regions of pancreatic endoderm. Remarkably, these two regions develop into the dorsal and ventral pancreatic buds through distinct differentiation programs; ultimately, the two buds fuse into a single organ upon rotation of the gut later in development.

As discussed earlier, ventral foregut development is heavily dependent on signals from the adjacent cardiac/lateral plate mesoderm (Kumar et al., 2003). Whereas Fgf and Bmp signaling from underlying mesoderm are crucial for liver development, their down-regulation or absence is equally important for specification of the ventral pancreatic endoderm (Jung et al., 1999). This effect appears to be dose dependent such that moderate levels of Fgf signaling specify liver endoderm and low levels allow for pancreas formation. Studies in explant culture systems show that Fgf diverts endoderm to a liver fate by down regulating the pancreatic transcription factor Pdx1 (Serls et al., 2005). Conversely, removal of Fgf results in the expression of pancreatic genes in the hepatic endoderm. Collectively, these results suggest that pancreas represents a “default fate” for ventral foregut endoderm (Deutsch et al., 2001). Studies in which the effects of the homeobox factor Hhex are blocked suggest that this dose dependent effect may be partially mediated by the length of time or proximity to the Fgf source (cardiac mesoderm) (Bort et al., 2004).

In contrast to the ventral pancreas, dorsal pancreatic development is mediated by a different specification program that is dependent on its

proximity to the notochord and dorsal aortic endothelium rather than cardiac mesoderm. Following gut tube formation the dorsal pancreas initially lays in close contact with the notochord. Early studies demonstrated that this contact was crucial for pancreas development, since notochord removal resulted in loss of dorsal pancreas specification and pancreas-specific gene expression (Kim et al., 1997). This did not, however, have an effect on ventral pancreas development. The effects of the notochord on pancreas specification appear to be mediated by its inhibition of Shh signaling (Hebrok et al., 2000). During development, Shh is widely expressed in the endoderm with the exception that it is specifically absent in pre-pancreatic domains. Inhibition of contact between the notochord and dorsal pancreatic endoderm resulted in ectopic expression of Shh in this region and subsequent failure in dorsal pancreatic development. Furthermore, treatment with the Shh inhibitor cyclopamine led to ectopic pancreas formation (Kim et al., 1997). The suppressive effects of the notochord were found to be due to its expression of Fgf2 and activin- $\beta$ B, each of which can suppress Shh in isolated endoderm cultures (Hebrok, 1998).

Following initial contact with the notochord, pre-pancreatic endoderm specification becomes increasingly dependent on endothelial factors as the endoderm is displaced away from the notochord by the fusion of the paired aorta. Disturbances in pancreatic-endothelial interactions results in a failure of dorsal endoderm to express pancreatic genes, a defect that can be rescued by subsequent co-culture with dorsal aorta (Lammert et al., 2001). The ability of the endothelium to control dorsal pancreas specification was later found to be due to its ability to promote survival in the underlying dorsal mesenchyme. This allowed for mesenchymal production of Fgf10, which maintains and expands the pancreatic progenitor pool by inducing expression of Ptfa1 (an early marker of pancreatic development) in the pancreatic endoderm (Jacquemin et al., 2006).

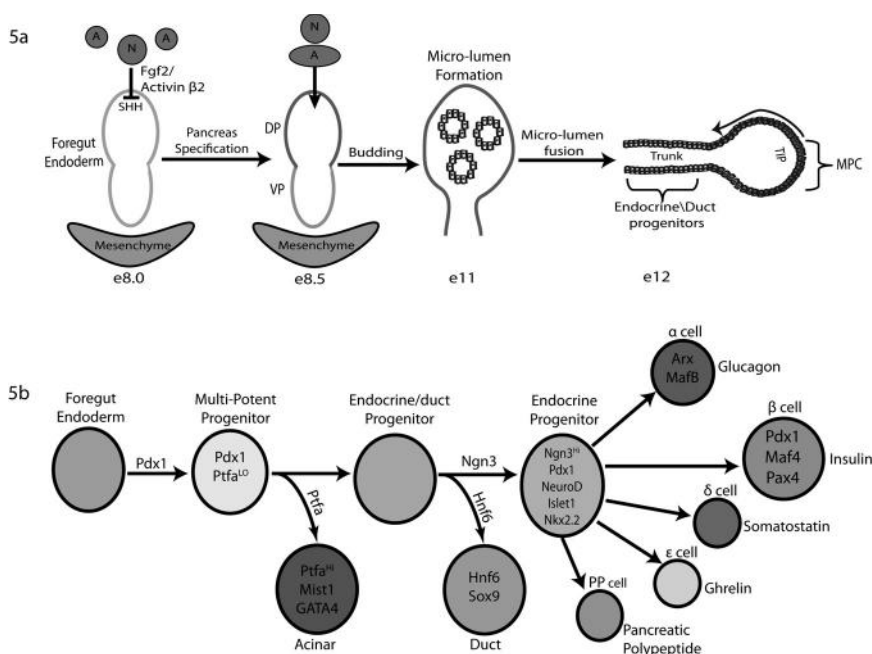
Transcriptional regulation of pancreatic specification involves a complex interplay between various signaling molecules, but the transcription factor network governing pancreatic development is primarily regulated through Pdx1 and Ptfa1 (Jonsson et al., 1994; Kawaguchi et al., 2002). Expression of these genes occurs at the onset of pancreas specification, with Pdx1 becoming detectable at E8.5 in pre-pancreatic, distal stomach and duodenal regions. Ptfa1 comes on slightly later and its expression is restricted to the pancreatic endoderm (Krapp et al., 1998). Genetic deletion of these transcription factors prior to specification results in pancreatic agenesis; Pdx1 mutants having additional malformations in the stomach and duodenum (Jonsson et al., 1994; Krapp et al., 1998). Conversely, ectopic expression of these factors results in expansion of the pancreas precursor population into more posterior endoderm (Afelik et al., 2006). Following specification, Pdx1 and Ptfa1 expression becomes increasingly restricted

to the endocrine (mainly  $\beta$ -cell) and acinar cell populations, respectively. As will be discussed later, this has important consequences for both morphogenesis and differentiation.

## **Pancreatic Morphogenesis**

Following establishment of the pancreatic endoderm, budding occurs first in the ventral region (E9) and is soon followed by a similar process in the dorsal region. Pancreatic bud growth is primarily driven by expansion of the multipotential pancreatic cells (MPC) that comprise the pancreatic progenitor pool and this expansion involves a combination of transcription factor activity and mesenchymal signaling. Relevant factors include Sox9, Hnf1 $\alpha$ /Tcf2 and downstream targets of the Notch signaling pathway (Seymour et al., 2007; Apelqvist et al., 1999), which are important for maintenance and proliferation of the MPC pool. Notch signaling in particular is thought to maintain MPC's in an undifferentiated state such that defects can lead to pancreas hypoplasia due to premature differentiation into endocrine cells. Regulation of MPC proliferation is also mediated by mesenchymal expression of Fgf10 and Bmp4/7, which as mentioned previously, exert their influence through modulation of endoderm transcription factors such as Ptf1a (Jacquemin et al., 2006; Murtaugh, 2007; Ahnfelt-Rønne et al., 2010).

The first wave of pancreatic development during bud development is characterized by the proliferation of the MPC pool and by formation of a distinct tubular network. The process by which these luminal structures develop and contribute to pancreatic growth is unique and essential to its proper development. In contrast to the classical "branching morphogenesis" seen in other branched organs, duct formation in the pancreas occurs in a more non-linear fashion (Villasenor et al., 2010). Initial formation of micro-luminal structures occurs following the random acquisition of polarity by cells in the unpolarized bud epithelium. The micro-lumens eventually fuse and remodel to form a mature pancreatic ductal system. In addition, during their formation, these tubular structures compartmentalize and specify the underlying pancreatic epithelium into distinct progenitor pools (Zhou et al., 2007). As these ducts expand, they appear to be composed of a rapidly expanding leading edge of "tip cells" and a trailing shaft of "trunk cells" that proliferate at a slower pace (Figure 5a). Lineage tracing experiments show that cells in these tip regions are likely MPC's that can give rise to all pancreatic cell lines whereas cells in the trunk regions result in primarily duct or endocrine populations. The importance of these tip/trunk regions to pancreas formation is further evidenced by experiments showing that disruption of tube formation leads to aberrant endocrine/exocrine development and morphogenesis (Schaffer et al., 2010; Solar et al., 2009; Seymour et al., 2007).



**Figure 5. mouse pancreas organogenesis.** (A) Schematic representation of pancreatic epithelial development and specification of acinar (red) and endocrine/duct (blue, green, respectively) lineages. Following fusion of microlumens and into tubular structures, formation of tip and trunk compartments occurs. Multi-potential Pancreatic Cells (MPC) along the tips take on an endocrine/ductal fate as cells migrate from tip to trunk regions. Between E13.5–14.5 the MPC become restricted to an acinar fate. (B) Transcriptional regulation of pancreatic development.  $\epsilon$  cells develop in the absence of Pax4 and Nkx 2.2 Adapted from Pan and Wright, 2011.

*Color image of this figure appears in the color plate section at the end of the book.*

## Pancreatic Differentiation

With continued expansion of the pancreatic bud, the embryonic pancreas enters into a second wave of development—often called the “secondary transition”—marked by expansion and differentiation of the epithelium into acinar, duct and endocrine cells. As mentioned previously, these cells arise from distinct progenitor populations within the tip and trunks of the tubular network. As the tip expands, cells at the leading edge eventually give rise to acinar units whereas those within the trunks give rise to endocrine and ductal cells. Although many of the transcription factors necessary for differentiation of these various cell types have been identified (Figure 5b), the nature of the intercellular signals that regulate this process, if any, are poorly understood.

## Acinar Differentiation

Pancreatic acinar formation becomes apparent at ~E13 of mouse development as the MPC's in the tip regions begin to differentiate into acinar cells. This transition is mediated through modulation of transcription factor expression such that the MPC's lose their multi-potential capabilities and take on an acinar fate. Initially these tip MPC's are maintained in a progenitor state through low level expression of various transcription factors including Pdx1 and Ptfa1. As tube expansion progresses, this expression pattern is modified such that Pdx1 is down regulated and Ptfa1 expression is increased, thus leading to MPC's taking on committed acinar fate (Schaffer et al., 2010; Solar et al., 2009; Seymour et al., 2007). This Ptfa1 dependent transition is primarily mediated by its association with the Rbp-J and Rbp-JL factors (Masui et al., 2010). During embryogenesis the Ptfa1/ Rbp-J complex is required to maintain MPC's in a progenitor state with disruption of this association leading to impaired pancreatic formation. As development progresses, Ptfa1/Rbp-J upregulates Rbp-JL leading to a feedback loop whereby Rbp-JL displaces Rbp-J, leading to increased formation of the Ptfa1/Rbp-JL complex. This switch allows for initiation of the acinar cell program which is further maintained by expression of transcription factors Mist1, Gata4, Nr5a2 and extrinsic regulation by Wnt/ $\beta$ -Catenin signaling (reviewed in Pan and Wright, 2011).

## Endocrine Differentiation

The endocrine pancreas is comprised of five cell types that secrete hormones responsible for regulation of glucose and nutrient hemostasis. Insulin producing  $\beta$  cells are the most prevalent cells within pancreatic islets, comprising 70–80% of all endocrine cells, with  $\alpha$ ,  $\delta$ , PP,  $\epsilon$  comprising the remainder.  $\alpha$  and  $\beta$  cells are responsible for glucagon- and insulin-mediated regulation of glucose, respectively. Pancreatic polypeptide-secreting PP cells and somatostatin-producing  $\delta$ -cells regulate exocrine pancreas and gallbladder function. Finally,  $\epsilon$ -cells secrete ghrelin, a hormone that stimulates the hunger response by acting on the central nervous system. Despite their different functions, these cell types arise from a common endocrine progenitor population that is specified in the trunk regions of the pancreatic tubular network.

As discussed earlier, the initiation of endocrine specification involves down-regulation of the Notch signaling pathway, which normally functions to maintain cells in an undifferentiated state. With repression of Notch, endocrine development is initiated as scattered cells in the trunk epithelium

begin to express the bHLH transcription factor Ngn3. Lineage labeling studies also demonstrate that all islet subtypes arise from this Ngn3<sup>+</sup> population (Gu et al., 2002). This factor that is central to specification of the endocrine pancreas—its absence leads to failure of endocrine compartment formation, whereas overexpression of Ngn3 results in re-direction of the pancreatic progenitor pool to an endocrine phenotype (Gradwohl et al., 2000; Jensen et al., 2000a; Schwitzgebel et al., 2000). In addition, Ngn3<sup>+</sup> endocrine progenitors activate Notch signaling in adjacent duct progenitor cells (by virtue of their expression of Notch ligand), leading to a feedback loop that limits the extent of endocrine differentiation and promotes morphogenesis (Magenheim et al., 2011; Jensen et al., 2000b).

Models of islet cell development suggest that Ngn3 is initially expressed at low levels, yielding a population of Ngn3<sup>LO</sup> cells that are endocrine-biased yet uncommitted. Subsequent asymmetric cell division results in Ngn3<sup>HI</sup> daughter cells that are endocrine committed and Ngn3<sup>LO</sup> cells which maintain the progenitor pool. Soon after asymmetric cell division, these Ngn3<sup>HI</sup> cells activate epithelial mesenchymal transition factor Snail2 and eventually migrate out of trunk epithelium to form the islets of Langerhans (Rukstalis and Habener, 2007). Importantly, islets are polyclonally derived from the progeny of multiple Ngn3<sup>+</sup> progenitor cells.

Islet subtype specification occurs soon after endocrine commitment as Ngn3 activates a host of transcription factors important for islet development. The initial cascade consists of a group of factors important for sustaining the islet precursor population and allowing for differentiation into appropriate subtypes. Such factors include Neurod1, Isl-1, Insm-1 and Rfx6 (reviewed in Gittes, 2009). Defects in expression of these factors can lead to a reduced number of endocrine cells and their subtypes. Following this initial step, lineage-specific factors are activated, resulting in the formation of the different islet cell populations. Central players in this process include Pax6, Pax4, Arx, Pdx1 and Nkx 2.2 (reviewed in Murtaugh, 2007). A complex interplay exists between these factors such that formation of each islet cell subtype requires the expression of certain factors and the absence of others. Additionally, alterations in transcription factor expression patterns does not lead to a reduction in the total number of endocrine cells but rather to altered ratios of the various subtypes. This concept is highlighted by the role of Pax4 and Arx in  $\beta$  and  $\alpha$ -cell development. Pax4 is a known key element in specification of  $\beta$  cells and  $\delta$  cells, while Arx is required for  $\alpha$ -cell formation. In Pax4 knockout animals there is a significant reduction in the number of  $\beta/\delta$ -cells, with likely shunting of this population towards  $\alpha$ -cell formation. Conversely Arx loss leads to absence of  $\alpha$ -cells with a concomitant rise in the number of  $\beta/\delta$  cell pre-cursors. This interplay



between Pax4 and Arx is in part mediated by the ability of Pax4 to act as a direct inhibitor of Arx expression (Collombat et al., 2003, 2005, 2007, 2009). Thus it appears that these two factors engage in a mutually repressive relationship which is required for appropriate allocation of precursors to  $\beta$  and  $\alpha$  cell identities.

Following the formation of individual islet precursors, these cells undergo a process of maturation to become functional endocrine cells capable of meeting the physiological demands of the post-embryonic pancreas. A hallmark of this process is that defects in factors responsible for maturation do not alter embryonic endocrine cell development but rather lead to defective postnatal function. Again, using the example of  $\beta$  cell development, we see that this maturation effect is regulated by a combination of newly expressed factors MafA/MafB and transcription factors used during initial lineage allocation (Aramata et al., 2005; Artner et al., 2006). During these later stages of pancreas development, immature  $\beta$  cells—along with their  $\alpha$  cell counterparts—initially express the bZip factor MafB (Artner et al., 2006, 2007). As  $\beta$  cells mature, they transition to a MafB+/MafA+ intermediate state and eventually become Maf-/MafA+ when fully mature (Artner et al., 2010). Similarly, the early islet specification factors NeuroD1 and Foxa2 (Naya et al., 1997; Huang et al., 2000; Sund et al., 2001) are also required for proper  $\beta$  cell maturation. Loss or disruption of any of the genes involved in the transition from an immature to a mature  $\beta$  cell state typically results in improper postnatal glucose homeostasis (Artner et al., 2007, 2010).

## Duct Development

Formation of the pancreatic ductal system is unique in that it results from the remodeling of a multi-luminal tubular plexus rather than through classic epithelial budding and tube extension (Villasenor et al., 2010). The end result is a series of terminal ducts which fuse to form intra then inter lobular ducts and eventually connect with the main pancreatic duct to empty into the duodenum. These ducts are lined by pancreatic ductal epithelial cells which are derived from the trunk progenitor pool as discussed previously. The exact mechanism and regulation of duct formation is not well understood but likely involves various aspects of the Hnf gene cascade and the early pancreas factors Pdx1 and Ptf1a (Pierreux et al., 2006; Hale et al., 2005; Kawaguchi et al., 2002). Mutations in these factors lead to significant ductal defects, although it is unclear if this a primary effect on duct epithelium or secondary to improper development of other pancreatic cell types. Further insight into the formation of pancreatic ducts and the junction between duct and acinar cells is likely to be important, as there is evidence to suggest

that duct epithelium in the terminal duct regions (centroacinar cells) may function as facultative stem cells during times of injury.

## **ORGAN SIZE CONTROL**

In spite of the centuries old observation that some tissues can fully recover their size after injury or partial removal, surprisingly little is known about how organs regulate size at a whole tissue level. How does an organ measure its mass? Is this process regulated or does it involve predetermined setpoints and mechanisms? How is growth controlled at the organ level? We have only limited insight into these fundamental questions of size biology and their answers will prove to be crucial in furthering our progress in fields including regenerative medicine and cancer.

Despite our incomplete understanding of the regulators of organ size, observations over the past century have provided insight into some of the key aspects of size determination. These studies demonstrate that the integration of cell size and proliferation are important in determining final organ mass and that there are both global and intrinsic mechanisms that regulate this process. In this section, we will review basic principles of size control and discuss how these mechanisms manifest in the regulation of liver size.

### **Sensing Tissue Mass**

Differences in size are often seen as one of the key characteristics that differentiate one organism from another. Between species, these differences seem to be accounted for by the variation in the total cell numbers. The human body, for example, contains significantly more cells than our murine counterparts (Baserga, 1985), which translates into a significant difference in size between the two. Thus a mechanism through which organ size is monitored by counting total cell numbers would seem most logical. Unfortunately the answer is not so simple.

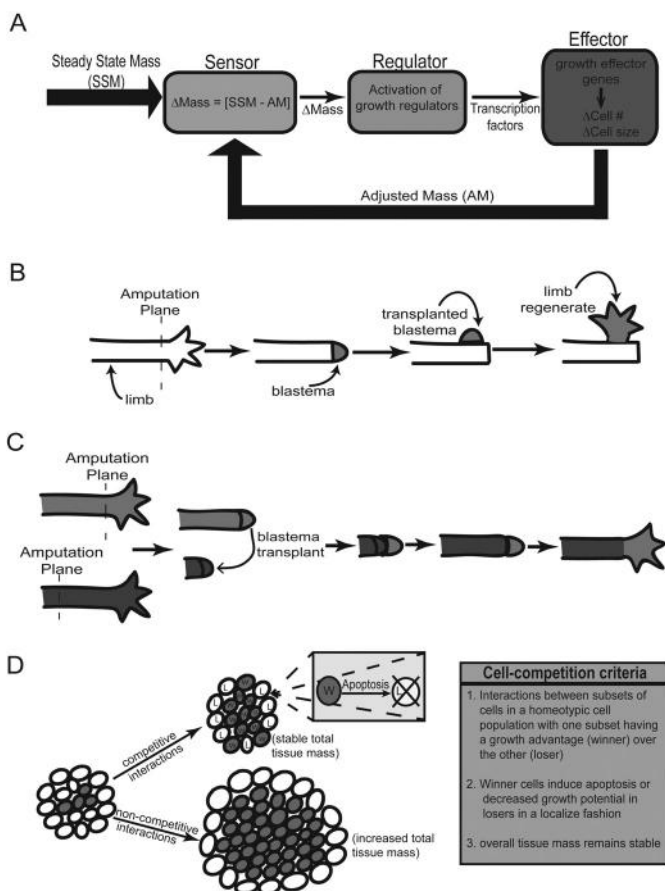
Experiments performed in salamanders in the mid 20th century suggested that total organ mass rather than cell size or number is being monitored (Frankhauser, 1945; Day and Lawrence, 2000). Because cell size and DNA content are co-regulated, changes in ploidy result in concomitant change in cell size (i.e., tetraploid cells are typically twice the size of diploid cells). By manipulating cell ploidy, it was observed that despite the near doubling in individual cell volume from diploid to tetraploid, the mass of each salamander and mouse embryo remained roughly the same (Frankenhauser, 1945; Henery et al., 1992). It is possible that size could be monitored through sensing of DNA copy number. However, this is

unlikely as other non-ploidy based methods of manipulating cell size such as embryo aggregation or altering blastomere number also did not affect final total tissue mass (Tarkowski 1959; Buehr and McLaren, 1974; Lewis and Rossant, 1982). Similarly, when cell number rather than cell size was altered in *Drosophila*, organ size appeared to remain stable. For example, mutations of *Drosophila* E2F (Neufeld et al., 1998) or Cdc2 kinase (Weigmann et al., 1997) cause an increase or decrease of cell proliferation in the wing compartment, respectively. In spite of these alterations in cell number, however, the wing dimensions in these settings remained relatively stable due to compensatory adjustments in cell size.

However, in some situations such as starvation or dysregulation of certain growth/proliferation pathways like Akt/PI3K (Verdu et al., 1999) or P27 (Ferro et al., 1996), changes in cell size or number are not entirely compensated for by such reciprocal changes. For example, manipulation of the Akt/PI3K pathway in *Drosophila*, resulted in changes in cell and compartment size that was independent of cell proliferation. This would suggest that organ size may increase or decrease through an apparent sensing of cell division or growth rather than total mass. Alternatively, instead of sensing size or number, such mutations may actually leave the mass sensing system intact but alter the size set-point by changing sensitivity to circulating/local factors or mitogens. If it is the case that total organ mass is sensed and adjusted via changes in cell size and/or number then the size control mechanism can be thought of operating in the fashion of a closed loop control system (i.e., linear feedback system). Akin to how a thermostat ascertains and regulates temperature, such a feedback-regulated system would sense its current mass (by measuring current organ functionality or tissue specific factors) and compare it to a value that is suggestive of its optimal size. The difference in mass would likely be relayed through activation of growth regulatory pathways resulting in adjustment of organ size by altering cellular proliferation and growth. In this manner organs could incorporate intrinsic and extrinsic cues such that mass is maintained within the given range necessary for homeostasis (Figure 6a).

## Extrinsic Regulation

On a global scale, systemic growth factors are likely key players in regulating total body size. Such extrinsic regulation is seen through the effects of various humoral factors, most notably growth hormone (Gh) and insulin like growth factor (Igf). In conditions of Gh excess, as seen in the syndrome of acromegaly, proportionate increase in total body size occurs, with the most pronounced features presenting in limbs and bony structures (Shlomo, 2006). Similarly, in genetic studies of canine size determinants, the size differences among various breeds were attributed to polymorphisms in the



**Figure 6. principles of organ size determination.** (A) Closed loop control system model for organ size regulation. Current mass of an organ is sensed by a sensor (blue) and compared to a value indicative of necessary organ mass required to maintain homeostasis. This difference in mass triggers activation of growth regulators (Regulator, orange) which leads to changes in cell size and number (Effector, maroon) in an attempt to re-establish appropriate mass. (B) Autonomously controlled salamander limb regeneration. When the blastema (red) at the site of amputation is transplanted to another location on the limb, it only regenerates the portion of the limb distal to the site of the original amputation. (C) Regulated control of salamander limb regeneration. When the blastema (red) from one amputated limb is transplanted onto a blastema (blue) that has formed at a different amputation site, the two blastemas communicate such that the transplanted blastema delays its regeneration until proximal limb formation has occurred. (D) Model of Cell competition. In competitive interactions, winner cells (red) increase in number at the expense of loser cells (white) through induction of apoptosis in the loser cells. This leads to increase in the winner:loser cell ratio but overall organ size is maintained. In non-competitive interactions the growth advantaged (red) cells increase in number but the number of wild type (white) cells does not decrease leading to an increase in overall organ size.

Color image of this figure appears in the color plate section at the end of the book.

Igf-1 locus (Sutter et al., 2007). Such circulating factors likely regulate total body size in response to nutrient availability and changes in environmental factors. But exactly how this regulation occurs at a tissue/organ specific level is not clear. Moreover, such circulating factors are unlikely to constitute a size “setpoint” as circulating levels of these hormones do not change dramatically at puberty, when growth ceases.

It may be possible that such factors mediate their effects through regulatory programs that are inherent to and specific for individual organs. In studies of Chuvash polycythemia, an autosomal recessive disorder involving the oxygen sensing mechanism, patients were noted to have enlarged liver, spleen and kidneys in addition to the increased RBC mass (Yoon et al., 2010). Mutations in the von Hippel-Lindau gene lead to defective degradation of HIF-1 $\alpha$  and -2 $\alpha$  resulting in an increased ratio of Hif-2 $\alpha$  to -1 $\alpha$  (Ang et al., 2002; Safran and Kaelin, 2003). On an organ specific level this translates into an increased suppression of the cyclin inhibitor p21<sup>cip1</sup> by Hif-2 $\alpha$ , thus leading to increased cellular proliferation and organ mass (Haase et al., 2001; Kim et al., 2006). Though these mutations are present in all tissues, only some organs are affected. This suggests that although humoral factors can regulate total body dimensions, size information is likely encoded/sensed on a tissue specific level and systemic factors modulate it within this framework.

### **Intrinsic Regulation (Autonomous vs. Regulated)**

This concept that intrinsic factors (i.e., mechanisms inherent to the tissue) function as regulators of tissue size initially arose from observations made during limb transplantation experiments performed over the past century. Some of the earliest such studies involved the reciprocal transplantation of limbs between the salamander *A. tigrinum* and its smaller cousin *A. punctatum* (Harrison, 1924). Surprisingly, despite the differences in size between the two species, determinants within the donor appeared to be the primary determinants of final limb size (Twitty and Schwind, 1931). Thus, at least in the case of the salamander limb, size and pattern information are encoded in an organ specific manner.

Such intrinsic control can be thought to function in one of two ways. It can occur in an autonomous fashion along a predetermined path that is independent of developmental context or it can be regulated wherein size is adjusted to fit within the framework of surrounding tissue. As discussed previously, systemic control of body size must occur in a coordinated fashion. Whether this is the case on a local tissue level is not clear but work done on amphibian limb regeneration appears to provide us some clues.

Following amputation of a salamander limb, regeneration occurs through the formation of a blastema or growth zone consisting of cells from the site of amputation that have re-entered the cell cycle (reviewed in Stocum and Cameron, 2011). The blastema, regardless of whether it is left in place or transplanted to another site, will only regenerate structures distal to its original position (Figure 6b) (Pietsch and Webber, 1965; Stocum, 1968). Thus, the blastema appears to be a self-organizing autonomous structure that carries within it all the information necessary to regenerate a limb to its appropriate size.

But autonomy is not without its limits. When the blastema from a distal amputation site is grafted to a proximal limb stump, formation of distal structures by the transplanted blastema is delayed until proximal limb formation is completed (Figure 6c) (Nardi and Stocum, 1983; Pecorino et al., 1996). This process, known as intercalary regeneration, suggests that blastema growth is also subject to regulatory control. How is it then that a usually autonomous process is subjected to regulatory control? It appears that this is accomplished through the ability of individual cells to sense and relay positional information to their neighbors. In the regenerating limb, cells within the growth zone are imparted with a memory of their position (Bryant and Gardiner, 1992; Brockes, 1997), which allows them to develop into appropriate structures upon exiting the blastema. But in the setting where discontinuities in positional information exist, these cells are capable of sensing the positional identity of their neighbors (through comparison of cell surface molecules) and adjusting blastema growth to re-establish appropriate limb morphology (da Silva et al., 2002). Thus, to an extent, the autonomous or regulated nature of size control is dependent on the context within which it occurs.

## **Cell Competition**

This ability for localized cellular interactions to influence total organ size is a process that is not unique to limb regeneration alone. Rather it appears that such mechanisms may in fact be evolutionarily conserved. Studies of *Drosophila* imaginal disc development provide us with insight as to how these may occur. Imaginal discs, clusters of cells that serve as the precursors for various organs such as the eyes, wings, etc., grow in a fashion that appears to be autonomous (e.g., they undergo a fixed amount of divisions from a set number of progenitors). But much like in the developing blastema, final organ size is also dependent on exchange of information between cells of the imaginal disc (Simpson, 1976). This becomes quite apparent when examining disc growth under the influence of mutations that affect

cellular proliferation, such as those seen in the *Drosophila* ribosomal proteins known as minutes (Morata, 1975). In mutant *Drosophila*, discs that are composed entirely of these slow growing cells will develop into smaller yet properly patterned structures. But in the mosaic setting, when discs are composed of both mutant and wild type cells, rather than forming smaller or disproportionately sized organs the tissue dimensions are similar to those seen in wild type *Drosophila*. This appears to be due to the ability of the growth advantaged wild type or “winners” cells to sense the presence of the growth disadvantaged mutant or “losers” cells and eliminate them through apoptosis to give a normal sized organ (Figure 6d) (Morata, 1975; Simpson and Morata, 1981). This process, termed cell competition, is also seen in other growth modifying mutations such as c-Myc, Hippo pathway components and Igl (de la Cova et al., 2004; Tyler et al., 2007; Menendez, 2010; Tamori et al., 2010). However not all mutations in the growth regulatory pathways lead to cell competition, as evidenced by the fact that alterations in the nutrient sensing AKT pathway or cell cycle regulator Cyclin D/Cdk4 result in non-competitive autonomous growth (Verdu et al., 1999; Bohni et al., 1999; Leever et al., 1996). The exact mechanisms through which cell competition is regulated and the question of how cells sense the “winner” or “loser” status of another, are not well understood. There is also limited evidence that size regulation through these types of cell-competitive interactions are conserved in mammals (Oliver et al., 2004; Ortel et al., 2006).

## ACQUISITION OF SIZE CONTROL PROGRAMS

Taken together, the above data suggests that regulation of organ size occurs in a manner that is guided by the following principles: (1) organs regulate size by sensing their total mass rather than individual cell numbers or size, (2) final tissue size can be determined in an autonomous or non-autonomous fashion that often involves integration of extrinsic and tissue specific (intrinsic) factors and (3) the processes that regulate size vary not only among different species but also between various tissues within a given organism. Studies involving fetal-to-adult transplants demonstrated that while some organs (spleen and liver) are subject to extrinsic growth regulation, other tissues (thymus, heart and kidney) grow according to an intrinsic size program (Metcalf, 1963, 1964). Recent data suggest that the basis for such differences is likely established during early embryonic development. By ablating progenitors in both embryonic liver and pancreas compartments, it was found that while the liver compensates and returns to normal size, the pancreas is restricted by the initial size of its progenitor

pool and remains small well into the postnatal period (Stanger et al., 2007). Such dependency on the number of progenitor cells is likely to exist in other tissues and may partly explain how growth occurs in an autonomous vs. regulated fashion.

## **Liver Size Control and Regeneration**

In vertebrates, the liver's ability to regulate size following regeneration may serve as a model through which we can answer these questions, since the liver is able to reproducibly return to its original size following injury. To date, many of the factors involved in regeneration have been uncovered but the mechanisms responsible for sensing and maintaining liver size remain unknown. Following removal of nearly 70% of its mass (2/3 partial hepatectomy), the remnant liver demonstrates a unique ability to undergo a period of rapid growth resulting in complete restoration of its original mass (Higgins, 1931). This process occurs through a series of coordinated steps and begins with activation of extracellular matrix remodeling by urokinase and matrix metalloproteinase's resulting in the release of Hgf (hepatocyte growth factor) that is bound to it. The ensuing increase in Hgf and other growth factors (Egf, Tgfa, Fgf, growth hormones and cytokines) is accompanied by activation of various cellular signaling pathways (Wnt, AKT, cyclin, etc.) leading to up-regulation of DNA synthesis over the next 24hrs (Michalopoulos, 2007). This "priming" phase is subsequently followed by replication of adult hepatocytes leading to complete restoration of liver mass in 5–7 days (Fausto, 2000). In conjunction with growth stimulation, the liver also releases anti-mitogenic factors such as Tgfβ1 and Activin A which correct for any over-shoot in liver size through apoptotic mechanisms (Houck and Michalopoulos, 1989; Ho et al., 2004; Sakamoto et al., 1999). While cellular proliferation of hepatocytes is important to the normal regenerative process, it is not absolutely necessary. In situations where hepatocyte proliferation is impaired, as seen in hepatotoxin injury, regeneration occurs via recruitment of facultative stem cells known as oval cells (Gerber et al., 1983; Factor et al., 1994). In non-injury settings, where hepatocyte replication is inhibited through extensive telomerase dysfunction, the liver is primarily reconstituted through an increase in size of the remaining hepatocytes following partial hepatectomy (Lazzerini et al., 2006). Regardless of the mechanism through which the tissue replacement occurs, the fact remains that under physiologic settings the liver always grows back to its original size.

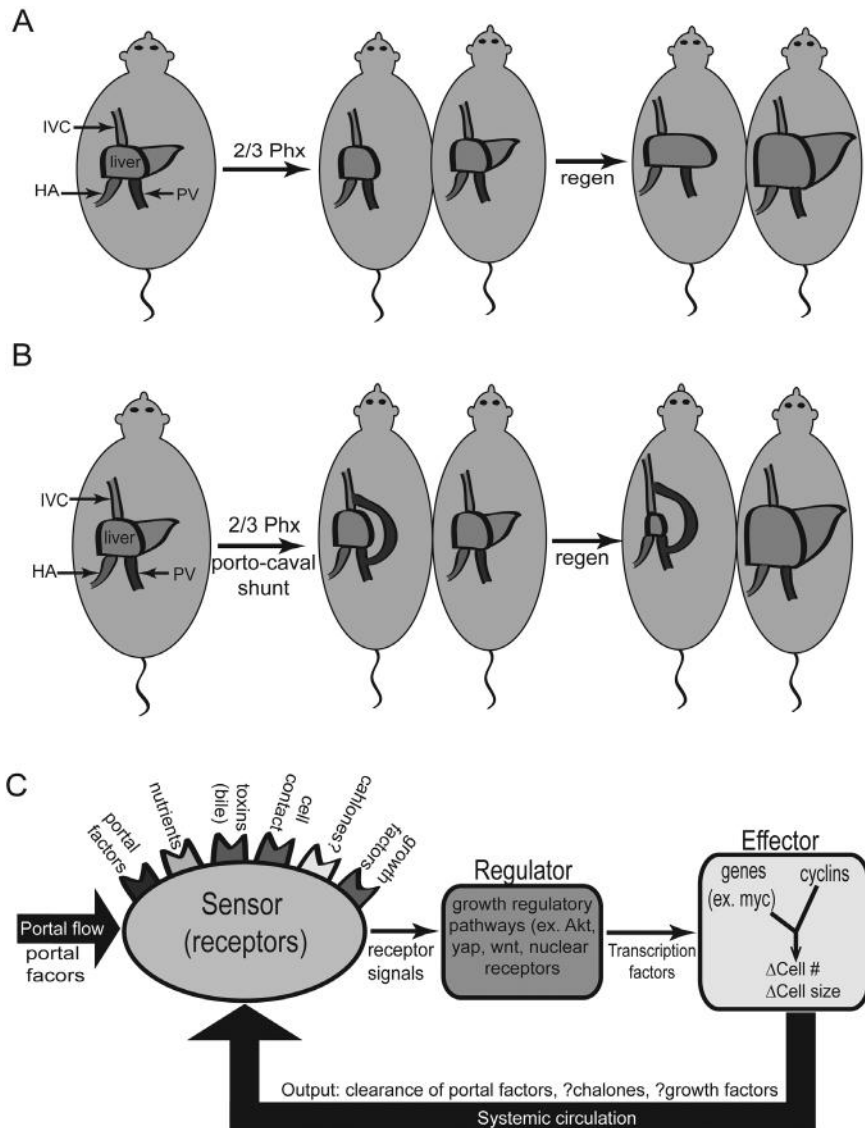


## Liver Growth Determinants

How then does the liver assess its mass and return to its appropriate dimensions? Although there is no clear answer at present, parabiosis experiments performed in the mid part of the last century provide a clue. By inducing cross-circulation between normal rats and those that had undergone varying degrees of partial hepatectomy, it was found that unaltered livers grew in response to unknown factors in the shared blood supply (Figure 7a) (Moolten and Bucher, 1967). It was hypothesized that this occurred as a result of either impaired clearance of these factors or decreased production of an inhibitory factor by the liver remnant. Later studies employing similar methods in the setting of total hepatectomy or porto-systemic shunts (diversion of portal blood flow from the liver into the inferior vena cava) suggested that systemic size-regulatory factors are likely stimulatory in nature and are present within the portal blood supply (Figure 7b) (Fisher et al., 1971). If indeed such regulatory factors exist, then changes in its levels should be (1) sensed and responded to by the liver in regenerative and non-regenerative settings and (2) correlated with changes in liver size such that the correct ratio of factor to liver mass is re-established.

Over the past two decades, examination of serum from various rodent partial hepatectomy models has resulted in the discovery of numerous circulating factors that are important in regulating liver growth and regeneration. Such factors, as mentioned previously, include Hgf, Egf, Tgf $\alpha$ , Fgf and the cytokines Tnf and Il-6. In various transgenic models these factors were found to both stimulate growth and increase final organ size. Although it is possible that the liver assesses its mass by sensing the levels of anyone of these stimulatory factors, knockdown/knockout experiments failed to reveal a deficit in the livers ability to return to its original dimensions following regeneration (reviewed in Michalopoulos, 2007). This would suggest that while such growth factors may be sufficient to induce changes in liver size, they are not necessary for the liver to sense and maintain its mass.

Alternatively, rather than measuring the level of stimulatory input, hepatic dimensions could be a reflection of inhibitory factors that are both produced and sensed by the liver. The precedence for the role of such "chalone" exists in the regulation of muscle mass by myostatin, an anti-mitogenic factor, which is both secreted and detected by muscles (McPherron et al., 1997; Mcpherron and Lee, 1997). Through a negative feedback mechanism, myocyte precursors sense the circulating levels of this factor and adjust their size accordingly. As a result, total muscle mass can be made to directly reflect the concentration of available myostatin. However, no such "hepatic chalones" have been found to date. As mentioned previously,



**Figure 7. regulation of liver size.** (A) Parabiosis of partial hepatectomized rat with a normal rat, leading to increased liver size of non-operated parabiotic partner. (B) Parabiosis of normal rat with a rat that has undergone partial hepatectomy and porto-caval shunt leads to increase in size of normal rat liver and decrease in size of partial hepatectomized liver. (C) Model of liver size regulation. The liver is able to detect various measures of its ability to perform normal homeostatic functions and in settings where differences are detected, size is adjusted to compensate.

Color image of this figure appears in the color plate section at the end of the book.

Tgfb $\beta$  and activin A can function as negative regulators of hepatic growth; however, their absence does not have an analogous overgrowth effect in the liver (Oe et al., 2004). Rather than acting as primary determinants of liver size, these positive and negative factors likely function as an adaptive mechanism invoked by the liver to rapidly adjust growth following injury, acting as regulators rather than sensors of size.

If detection of growth regulators released during regeneration is not the primary method by which the liver takes account of its size, then as the parabiosis studies suggest, mass may actually be sensed instead through clearance of portal factors by the liver. Although relatively little is known about these factors, recent studies point to bile acids as key to this process (Wendong et al., 2006). Following secretion of bile into the intestines by the liver, it is reabsorbed via enterohepatic circulation and subsequently removed from portal flow by hepatocytes. By manipulating the levels of bile acids, it was found that while increased levels potentiated liver growth during regeneration, low levels or disruption of bile sensing by its nuclear receptor, FXR, delayed growth recovery. Thus, the liver may take measure of mass through its ability to carry out normal homeostatic processes rather than through the detection of chalone or endogenous growth factors (Figure 7c).

## **Liver Growth Regulators**

Although bile acids are clearly important for regulation of hepatic size, it is unlikely that they are the sole mechanism by which the liver can sense and adjust its mass. Another regulator of liver size is the PI3K/Akt/mTOR pathway which functions as a key sensor of nutrient and insulin dependent homeostasis (Brazil and Hemmings, 2001). In pathologic settings its aberrant activation results in increased cell size and liver mass (Ward, 1997; Ono et al., 2003; Shioi et al., 2002). Likewise, defects in the tumor suppressor Pten (a negative regulator of the Akt pathway) leads to hepatomegaly (Stiles et al., 2004). In addition to its growth promoting activities, Akt serves a vital role in the maintenance of liver size. In the setting of partial hepatectomy, absence of Akt signaling not only impairs hepatocyte growth but can also lead to death from an inability of the liver to regain sufficient mass (Chen et al., 2001; Hong et al., 2000; Haga et al., 2005).

Similarly, mechanisms responsible for sensing cellular interactions have also proven to be indispensable in regulation of hepatic size. The Wnt/ $\beta$ -catenin pathway is well known for its role in cell-cell communication, cell polarity and proliferation. Liver specific knock-outs of  $\beta$ -catenin demonstrated a 15–25% reduction in hepatic mass whereas overexpression led to a 20% increase in size (Sekine et al., 2007; Nejak-Bowen and Monga,

2008; Harada et al., 2002). Interestingly, in response to changes in  $\beta$ -catenin, expression levels of various metabolic enzymes such as glutamine synthetase (Glu1) and Cytochrome P450's were also altered. Thus in response to changes in cellular interactions, Wnt/ $\beta$ -catenin signaling may affect liver size through alterations in both cell proliferation and sensitivity to various metabolic stimuli.

More recently, the Hippo/Yap pathway was discovered as an important sensor of cell-cell interactions and regulator of tissue mass (Dong et al., 2007; Pan, 2007). Under normal conditions, the growth promoter Yap is phosphorylated and sequestered in the cytoplasm by Hippo signaling. This functions to restrict organ size such that on release of tonic inhibition, Yap translocates to the nucleus, resulting in proliferation and widespread tissue growth. In the liver this leads to hepatomegaly and eventual formation of hepatocellular cancer (Camargo et al., 2007). More importantly this pathway appears to be vital in regulating growth through cell contact inhibition (Zhao et al., 2007; Ota and Sasaki, 2008). In culture systems, high cell density leads to hippo induced Yap inactivation, whereas Yap up-regulation can overcome this effect and resulting in cell overgrowth. In this manner, Hippo/Yap signaling may coordinate local intercellular information into the regulation of whole organ size.

It is likely that these different mechanisms for sensing metabolic state and local cellular environments work together to coordinate organ growth. While it is unclear how this occurs, experiments examining the patterns of cyclin activation and genetic targets provide some clues. In studies of cardiac size regulation it was found that Yap can complex with  $\beta$ -catenin to induce cardiomyocyte proliferation and co-regulate heart growth (Heallen et al., 2011). Similarly in cell cycle studies, these factors were able to activate mitotic progression through cyclin D and E, such that in their absence the growth effects of Akt, Wnt/ $\beta$ -catenin and Yap are severely diminished (Mullany et al., 2007; Nelsen et al., 2002; Nejak-Bowen and Monga, 2008; Cao et al., 2008; Septer et al., 2011). While it appears that integration of these various growth modulating pathways may occur at the level of transcriptional and cell-cycle control, it is still not known exactly how they interact to form a network that relays size information to the entire organ.

## **Pancreatic Size Control**

In contrast to the liver's robust regenerative and size regulatory capabilities, the pancreas's size control mechanisms appear to be much more limited (Khalaileh et al., 2008). As we have mentioned previously, pancreatic size is determined in an autonomous fashion very early on during gestation (Stanger et al., 2007). This process is highly dependent on the number of progenitor cells present at the time of organogenesis such that their depletion

leads to a smaller pancreas at birth. The various pancreatic components, including the beta cell compartment, are subject to this method of size regulation in the embryonic period. Following the initial appearance of  $\beta$ -cells in the fetus, subsequent cells arise from a previous insulin negative population of  $\text{ngn3}^+/\text{pax4}^+$  cells (neogenesis) (Gu et al., 2002; Gradwohl et al., 2000). If this process is not given enough time to complete, as seen in intrauterine growth retardation (IUGR) or fetal malnutrition, then  $\beta$  cell mass remains insufficient into the postnatal period leading to eventual glucose intolerance and type 2 diabetes (Simmons et al., 2001; Garofano et al., 1997).

However this strictly autonomous growth does not necessarily mean that pancreas or  $\beta$  cell mass is fixed at birth and unable to change in postnatal development. In mouse models of pancreatic injury, as seen in  $\beta$  cell specific ablation or physical insult through partial pancreatectomy,  $\beta$  cell mass appears to increase soon after the damage has taken place (Nir et al., 2007; Pearson et al., 1977; Dor et al., 2004). Although this process is insufficient to replace the lost tissue mass (unlike in the liver), it still indicates that the size of the  $\beta$  cell compartment is subject to non-autonomous growth. Similarly in pregnant mice,  $\beta$  cell mass increases nearly 2.5 fold during pregnancy and rapidly returns to normal post-partum through apoptosis (Van Assche et al., 1980). Lineage labeling studies suggest that in the physiologic setting, increases in mass overtime are driven by replication from pre-existing  $\beta$  cells (Dor et al., 2004; Teta et al., 2007). While replication from differentiated cells is the primary mode of  $\beta$  cell regeneration,  $\beta$  cells arising from facultative progenitor cells has also been described, a process reminiscent of the proposed oval cell model of liver regeneration. In the setting of ligation of the main pancreatic duct, it was found that  $\beta$  cell mass increased proximal to the obstruction with cells primarily derived from  $\text{ngn3}^+$  progenitors originating from the ducts (Rosenberg, 1998; Xu et al., 2008; Dor and Melton, 2008).

Thus, it seems that while embryonic size regulation was entirely dependent on numbers of fetal progenitor cells, postnatal growth shifts to a liver type model wherein size control is guided by inputs affecting replication of differentiated cells. While it is unclear why or how this transition happens, it can be postulated that in the embryonic phase glucose homeostasis is mainly regulated by maternal factors, but in the postnatal period endogenous  $\beta$  cells are placed under increasing demand to maintain glucose homeostasis and thus adapt by developing mechanisms to adjust growth based on systemic inputs. This is reflected in recent findings suggesting that  $\beta$  cell mass is regulated by glucose metabolism (Porat et al., 2011). Using islet cell transplantation and  $\beta$  cell specific ablation techniques, the authors found that  $\beta$  cell mass is a function of the demand placed on individual  $\beta$  cells to produce insulin. In this manner, any loss in  $\beta$  cell

mass is sensed by each member of the compartment as increased glucose flux and leads to increased insulin production and cellular replication to maintain euglycemia. It has also been suggested that  $\beta$  cell replication may be influenced by neural inputs, although this still remains an area of controversy. Other growth factors (ex. Glucagon-like-peptide 1, Egf, gastrin) and metabolic signaling pathways such as Akt/mTOR have also been implicated in  $\beta$  cell mass regulation (Rooman and Bouwens, 2004).

## CONCLUSIONS

From the formation of gut endoderm to the appearance of distinct organ structures, the development of the liver and pancreas occurs through the complex interplay between various signaling pathways. These processes are not only important for proper embryonic growth but are also needed to establish correct organ pattern and size. We have already seen how recapitulation of these developmental signals can have important consequences on liver growth and regeneration. It is likely that these embryonic size programs are translated into the adult setting and are reflected in the manner by which different organs regulate their growth. In the adult liver, size is regulated in a non-autonomous fashion and appears to depend on the presence of portal factors and local cellular interactions (Figure 7c). The pancreas, on the other hand, primarily grows in an autonomous fashion with its final organ size determined during embryonic development. While we are beginning to understand the roles that stem cells, growth factors and homeostatic functions play in regulating organ size, we have yet to develop a clear understanding of how these various factors are integrated by different organs to establish specific size control programs. With recent advances in tissue engineering, stem cell and developmental biology, we now have the tools that may finally lead us to an understanding of how organ size is determined.

## ABBREVIATIONS

|         |                                   |
|---------|-----------------------------------|
| GI      | gastrointestinal                  |
| AIP     | anterior intestinal portal        |
| CIP     | caudal intestinal portal          |
| A-P     | anterior-posterior                |
| STM     | septum transversum mesenchyme     |
| BEC     | biliary epithelial cells          |
| I/E-HBD | intra/extra-hepatic biliary ducts |
| MPC     | multi-potential pancreatic cells  |

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**PART III**

**MODEL ORGANISMS**

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# Developmental Regulation and *De Novo* Formation of Stem Cells in Plants

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## SUMMARY

Through convergent evolution, both plants and animals have come to rely on stem cells to generate and maintain body plans. Many of the mechanisms required for the regulation of stem cells are similar between plants and animals. This chapter highlights the current state knowledge about stem cell regulation of plant development and *de novo* formation of stem cells during regeneration and lateral organ formation. Studies discussed specifically focus on two classic plant model organisms, *Arabidopsis thaliana* (Arabidopsis) and *Physcomitrella patens* (Physcomitrella). Arabidopsis and Physcomitrella are land plants that are part of the angiosperm and bryophyte (moss) families, respectively. Both types of plants have a morphological

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*List of abbreviation after the text.*

region called the meristem where stem cells reside. In *Arabidopsis* two meristems are responsible for the formation of the shoot and the root. These meristems are multiplex and contain multiple stem cells as well as multiple supporting niche cells. In contrast, *Physcomitrella* contains multiple meristems, but they are simplex with single stem cells and no identified niche cells. Despite the different types of meristems, both *Arabidopsis* and *Physcomitrella* utilize evolutionarily conserved homologous proteins to specify and maintain these populations. In addition, *Arabidopsis* and *Physcomitrella* have been extensively utilized to study regeneration and the *de novo* formation of stem cells. In *Arabidopsis*, regeneration has been studied by ablation or excision experiments, and the hormone induction of a plant-specific intermediate called a callus. A callus is an unorganized mass of cells that has the potential to regenerate an entire plant. *Arabidopsis* also has an inherent mechanism of *de novo* stem cell formation that is required for lateral root emergence. For *Physcomitrella*, regeneration is achieved via excision of any tissue, even a single isolated cell. In contrast to regeneration in *Arabidopsis*, *Physcomitrella* regeneration does not require induction by exogenous hormones or the formation of a disorganized callus. Thus, plants provide a tractable model system in which to advance our understanding of stem cell regulation, both developmentally and during regeneration or reprogramming. The developmental regulation, regeneration and *de novo* formation of stem cells in plant systems is described in this chapter.

## INTRODUCTION

Long before the assessment of stem cell function in animals and the controversies surrounding human stem cell research, biologists were investigating the role of stem cells in plants. Both plants and animals have evolved the ability to grow and regenerate cells or tissues through the asymmetric division of stem cell populations. Despite an evolution separated by 1.6 billion years, both plants and animals have converged on similar mechanisms of stem cell regulation. As a result of these common mechanisms, studies in plants have been important to inform research into animal systems and human disease states in general. For example, 70% of the genes implicated in human cancers are conserved in the model plant, *Arabidopsis thaliana*, and 71% of the genes implicated in neurological disorders are conserved (Jones et al., 2008; Xu and Møller, 2011). Thus, from a cell biological perspective, plant research is important to our understanding of the complex human disease state.

Using plants as a model system to study stem cells is advantageous for many reasons. The basic set up and maintenance required for plant research is relatively simple, and the costs associated with cultivation are low. Their amenability to transformation for the production of transgenics and the

large number of mutant and genomic resources also make plants a good model organism for stem cell studies. Plants are unique among multicellular organisms in that they are continually developing and undergoing stem cell renewal throughout their life cycle, thus they can provide a complete developmental overview within a single snapshot of time. As the parallels between plants and animals continue to be revealed, the study of convergent stem cell evolution in plants will become increasingly important for our understanding of stem cell regulation. This chapter is designed to provide an overview of the studies on stem cell specification and maintenance, and to discuss the burgeoning studies on stem cell-mediated regeneration in plants.

## PLANTS AS MODEL ORGANISMS

The green plant kingdom is vast and diverse, encompassing over 300,000 species and still counting (Judd et al., 2007). Research involving many different species has been conducted, however, our discussion will focus on two classic plant model organisms: *Arabidopsis thaliana* (hereafter, *Arabidopsis*) and *Physcomitrella patens* (hereafter, *Physcomitrella*).

*Arabidopsis* is a small, flowering plant (angiosperm) of the mustard family. For the past 30 years, *Arabidopsis* has been the model organism of choice for a multitude of plant studies. *Arabidopsis* was chosen because of its short generation time, simple structure and small genome. When the genome of *Arabidopsis* was sequenced in 2000, its significance as the basis for plant studies was cemented (*Arabidopsis* Genome Initiative, 2000).

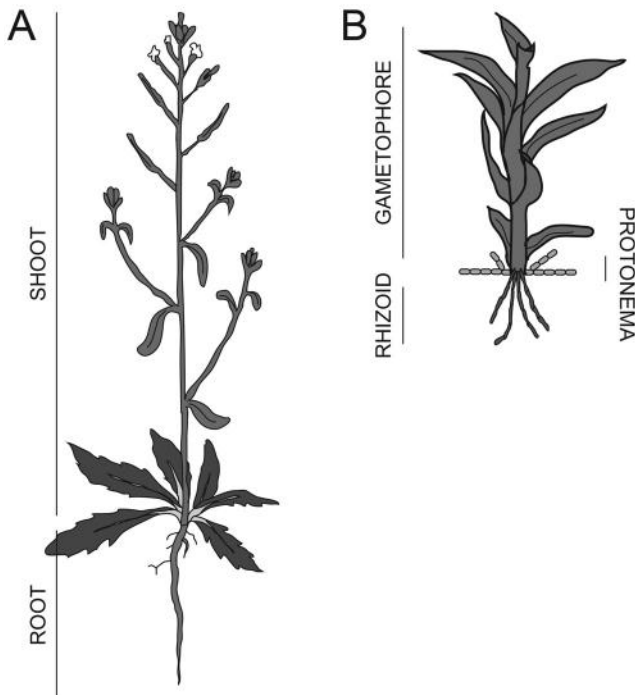
*Physcomitrella* is a moss that has been used extensively for evolutionary and developmental biology studies because of its ability to undergo homologous recombination and the physiological and developmental traits it shares with angiosperms. More recently, *Physcomitrella* has been used to study regeneration and the *de novo* formation of stem cells by reprogramming (Ishikawa et al., 2011; Nishiyama et al., 2012). Within this section we will provide a basic overview of plant biology and stem cell regulation in both *Arabidopsis* and *Physcomitrella*.

## Life Cycle and Body Plan of Land Plants

Land plants can be divided into seven main groups (in order of evolutionary divergence): liverworts, mosses, hornworts, lycopods, monilophytes, gymnosperms and angiosperms (Judd et al., 2007). Although each of these plants has a haploid-diploid life cycle, depending on the group, each spends the predominant amount of their life in one state. The two main classes of

plants discussed in this chapter, mosses and angiosperms, diverged early in the evolution of land plants. *Arabidopsis*, an angiosperm, has a diploid (sporophyte)-dominant life cycle, whereas *Physcomitrella* is haploid (gametophyte)-dominant. Another important distinction is that *Arabidopsis* is a vascular seed plant and *Physcomitrella* is seedless and non-vascular.

The basic architecture of *Arabidopsis* consists of shoots, the aboveground organs and roots, the belowground organs. The adult body plan originates from the plant embryo developed within a seed. During embryonic development, two distinct stem cell niches are established to serve the shoot and root respectively (Figure 1A). Similarly, the basic body plan of *Physcomitrella* includes leafy shoots and root-like tissues. However, unlike *Arabidopsis*, the leafy shoot (gametophore) is formed after hypha-like branching of a filamentous tissue (protonema) arising from a spore in a haploid generation. The root-like tissue (rhizoid) develops from the epidermis of this gametophore (Figure 1B). Each of these structures arises from a distinct stem cell.



**Figure 1. Gross morphology of *Arabidopsis* and *Physcomitrella*.** (A) In *Arabidopsis*, plants are divided into the above ground or shoot portion and the below ground or root portion. (B) In *Physcomitrella*, three morphological regions are defined. The protonema, or hypha-like structure forms first and produces a leafy shoot called the gametophore. In turn, the gametophore produces root-like structures called rhizoids.

In both types of plants, the morphological area encompassing the stem cell niche is called the “meristem”. A meristem consists of either multiple stem cells, as is the case with *Arabidopsis* or a single stem cell, as with *Physcomitrella*. Despite the differences in architecture between *Arabidopsis* and *Physcomitrella*, some striking similarities remain in the regulation of stem cells. The specification and maintenance of these cells and the potential for reprogramming are discussed below.

## **STEM CELL POPULATIONS IN PLANTS**

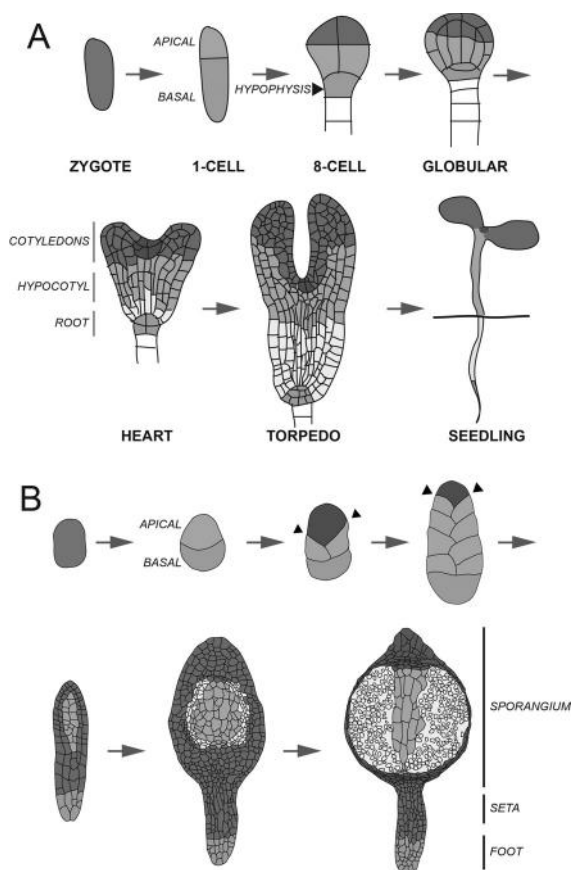
### **Embryonic Development**

Embryonic development of angiosperms occurs within the seed, and begins much like mammalian development with a single asymmetric division leading to embryonic and extra-embryonic cell fates. However, unlike animals in which embryonic development leads to a smaller version of the adult and fixed organ formation, after plant embryogenesis the result is a morphologically distinct juvenile stage (seedling), with adult organs forming post-embryonically. It is important to note that plants do not have a fixed body plan, and instead develop continually adapting to the environment around them. Although plants are highly adaptable, the importance of embryonic development should not be diminished. It has been revealed that apical-basal polarity is established during embryonic development and is critical for the formation and proper development of adult organs.

In *Arabidopsis*, the pattern of cell division during embryogenesis is a well-documented and stereotypical process. Initially, the zygote elongates and divides asymmetrically to produce a small apical cell and a larger basal cell (Figure 2A). Subsequently, the apical cell undergoes three more rounds of cell division to give rise to an eight-celled sphere. The upper and lower portions of this sphere will give rise to the apical and majority of the basal portions of the seedling respectively. Successive divisions within the eight-cell apical sphere lead to an inner and outer eight-cell layer, followed by an approximately 100-cell “globular” stage, and then an approximately 200-cell “heart” stage. At the “heart” stage of embryogenesis, morphologically distinct features of the seedling become apparent. Following the “heart” stage, embryos pass through the “torpedo” stage. It is in these later stages of embryonic development that the shoot meristem becomes identifiable.

In contrast to the apical cell, the larger basal cell divides horizontally to produce the “suspensor”. The development of the suspensor is less stereotypical, with the majority of the suspensor acting as extra-embryonic, maternal support. However, the upper-most cell of the suspensor, the hypophysis undergoes a stereotypical series of divisions to give rise to the





**Figure 2. Embryonic/Sporophyte Development of Arabidopsis and Physcomitrella.** (A) In Arabidopsis embryonic development begins with an asymmetric division of the zygote to produce an apical (light green) and basal (orange) cell in the 1-cell stage. Subsequent cell divisions of the apical cell result in an 8-cell stage (4 more cells behind what is depicted). At the 8-cell stage, the apical cells can be separated by the fates they will give rise to—dark green the cotyledons and light green the hypocotyl. In addition, the upper most cell of the basal lineage (hypophysis) is generated. The next stages are globular and heart. During the heart stage, cells fated to become the apical meristem are distinguishable (red). Additionally, the lower portion of the apical cells (yellow) and the hypophysis (orange) are distinguished as giving rise to the root. In the subsequent stage, torpedo, the cell layers are maintained to produce the embryonic seedling. Colors mark final location of shoot and root meristems, and developmental lineages. (B) Physcomitrella sporophyte development begins with an asymmetric division of zygote to give rise to apical (light green) and basal (orange) cell fates. Subsequent cell divisions result in a mature sporophyte consists of three parts: sporangium (capsule indicated in blue, spores indicated in yellow, and columella indicated in light green), seta (dark green), and foot (orange). Sporophyte apical cells are indicated in red. Arrowheads indicate planes of cell division. Original pictures for Physcomitrella drawings were provided by Dr. Keiko Sakakibara.

Color image of this figure appears in the color plate section at the end of the book.

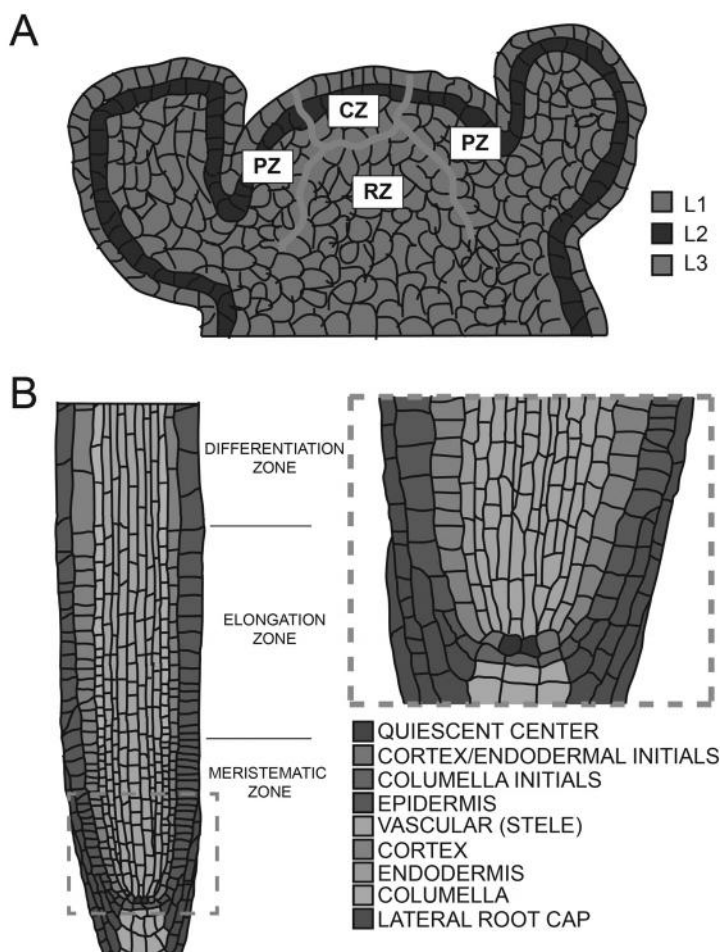
root meristem. Thus, early in embryonic development, both the root and shoot meristems are specified and organized (for a comprehensive review of *Arabidopsis* embryogenesis, see Capron et al., 2009).

*Physcomitrella* embryo development is similar to that of *Arabidopsis* at the early stages. At the first cell division of the zygote, an apical cell and a basal cell are formed (Figure 2B). The apical cell divides obliquely at two division planes to form two rows of wedge-shaped cells. In contrast to the indeterminate activity of embryonic stem cells in *Arabidopsis*, in *Physcomitrella*, the apical cell stops dividing after approximately 12 divisions. After these divisions, the wedge-shaped cells expand and divide periclinally to form inner and outer cell layers, which mainly differentiate into the sporogenous cells and sporangium capsule cells, respectively. The basal cell divides several times to form the basal-most cells of a foot which functions with the gametophyte cells to absorb nutrients; no stem cells are formed in this region (Kofuji et al., 2009). In contrast to *Arabidopsis*, the *Physcomitrella* stem cells and body plan established during embryogenesis are only necessary for spore production via meiosis, and this information is not transmitted after spore germination.

## **Meristem Organization and Stem Cells of the Post-Embryonic *Arabidopsis* Plant**

As mentioned previously, during post-embryonic development there are two distinct meristem populations in *Arabidopsis*, the shoot meristem and the root meristem. The shoot meristem gives rise to the aboveground organs: the stem, leaves and flowers, and can be divided into overlapping developmental layers and zones each with distinct properties. The uppermost layers of the meristem, L1 and L2, are each a single sheet of cells that divide within the plane of this sheet. In contrast, the lower layer, L3 is characterized by cell division in all planes (Figure 3A) (Satina et al., 1940).

The three zones of the shoot meristem have been defined as: the central zone, peripheral zone and rib zone. The central zone is located in the upper center of the meristem, with the peripheral zone surrounding it in the same plane, and the rib zone lying underneath (Figure 3A). The central zone contains the shoot stem cells, also called initial cells, which are divided among the layers (Stewart and Dermen, 1970; Schnittger et al., 1996). Upon division, cells are displaced into the surrounding peripheral zone or underlying rib zone where they undergo expansion and differentiation. Cells displaced into the peripheral zone will form leaf primordia, whereas cells in the rib zone will contribute to the formation of the stem. During the formation of leaf primordia, the contribution of cells from the three layers is



**Figure 3. *Arabidopsis* Meristems.** (A) The shoot meristem in *Arabidopsis* gives rise to all of the above ground organs. It can be divided into three layers: L1 (red), L2 (Blue), and L3 (grey) and three zones: Central Zone (CZ), Peripheral Zone (PZ) and Rib Zone (RZ). Light blue line indicates separation of zones. (B) The root meristem in *Arabidopsis* gives rise to the below ground organs. The root has radial or bilateral symmetry and can be divide into three developmental zones longitudinally beginning at the root tip: Meristematic Zone, Elongation Zone and Differentiation Zone. The Meristematic Zone contains the stem cells and niche cells, as indicated. Panel on the right is enlarged from the blue dashed box on the left.

*Color image of this figure appears in the color plate section at the end of the book.*

stereotypical. Cells from an L1 origin generally give rise to the epidermis, L2 to subepidermal cells (ground tissue and germ cells) and L3 to vasculature (Stewart and Dermen, 1970). Underlying the central zone, at the top of the rib zone lies a population of cells termed the organizing center. These cells

are thought to support and coordinate the behavior of surrounding initial cells (Mayer et al., 1998).

An interesting feature of the shoot meristem is the ability to change the type of organs being generated. For example, after germination, the *Arabidopsis* shoot meristem is first responsible for generating a rosette of leaves. Based on the perception of environmental cues, a developmental switch is flipped in the shoot apical meristem, and it begins to produce inflorescence (flower) meristems along with cauline leaves and stem. As the plant grows upward, post-embryonic meristems (axillary meristems) develop at the axils of leaves to form lateral branches. The axillary meristems are morphologically identical to the shoot meristem, and form lateral organs of the shoot (Clark et al., 1993; Endrizzi et al., 1996; Laux et al., 1996). Thus, the shoot meristem is a complex network of cells that requires precise coordination and repurposing to produce the aboveground organs throughout the *Arabidopsis* life cycle.

In contrast, the root meristem has a relatively simple organization, with a foundation of four cells in the center of the meristem called quiescent center cells. These cells, true to their name, remain largely quiescent during the *Arabidopsis* life cycle, and act as supporting cells in the root meristem (Clowes, 1957; Dolan et al., 1993). Surrounding the quiescent center are the root initial cells. There are four different initial cell types within the root meristem that give rise to distinct cells within the root architecture (Figure 3B) (Dolan et al., 1993). The four types of initial cells give rise to: i. the cortex and endodermis, ii. the stele (the vascular tissue plus pericycle), iii. the epidermis and lateral root cap and iv. the columella. Each initial cell type will divide asymmetrically to maintain the initial and give rise to a daughter cell. One example of the stem cell division and subsequent differentiation is the cortex/endodermis initial (CEI) cell. In this instance, the resulting daughter cell undergoes an anticlinal division (perpendicular to the mother cell) to produce two cell layers, the cortex and endodermis. The cortex and endodermis cells then proceed through distinct developmental phases; first, the cells rapidly divide in a transit-amplifying phase within the meristematic zone. After rapid division, the cells enter the elongation zone and lengthen along their longitudinal axis. Following cell elongation, there is entrance into the differentiation zone where cells acquire their specialized features and functions.

Similar to the shoot, there is lateral organ development in the root as well, marked by the emergence of lateral roots. However in contrast to the shoot, lateral root primordia are not established from the root meristem, but instead form *de novo* later in development. A recent study provides evidence that a molecular signature for lateral root formation is initiated in a region of the root tip called the oscillation zone (late meristematic/early elongation zone), but the morphological appearance of lateral primordia

is not apparent until the differentiation zone (Moreno-Risueno et al., 2010). The formation of lateral roots will be discussed in detail later.

While initial cells are named based on the cells they eventually give rise to or the region they originate from, the general developmental plasticity of plant cells means these initial cells are actually pluripotent. Thus, at least theoretically, stem cells have the ability to become any cell in the plant. The positional and molecular signals contributing to cell fate decisions in the meristem are the topic of discussion within a later section of this chapter.

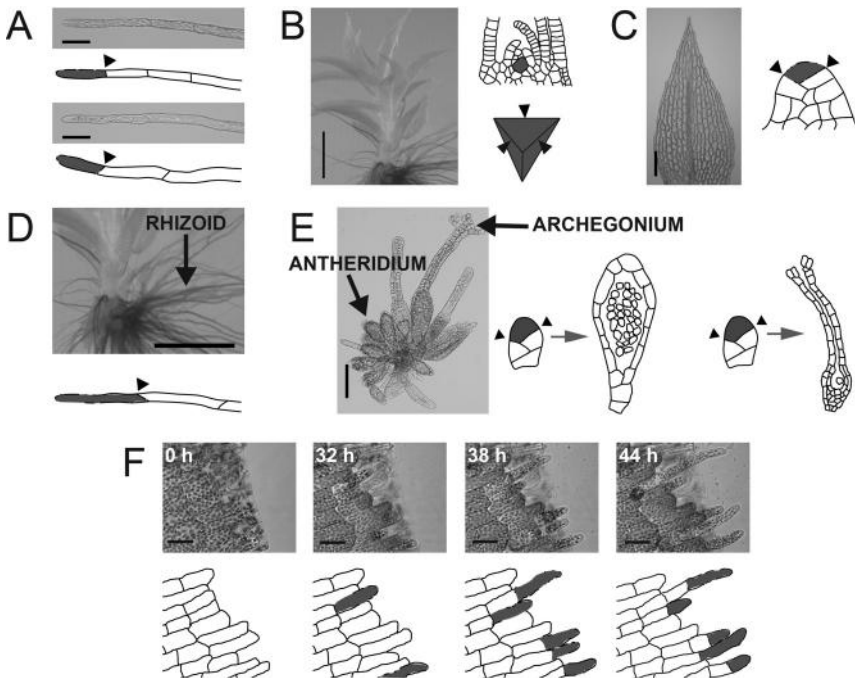
Taken together, both shoot and root meristems contain initial cells with an underlying cellular support niche. However, the shoot meristem is more complex, giving rise to multiple organs and altering the production of these organs during the *Arabidopsis* life cycle. Despite the differences between shoot and root meristems, the plasticity of plant cells remains constant, and studies have revealed that under the correct conditions, root meristems can be transformed into shoot meristems (Gallois, 2004). Thus the cell fate of plant cells is not fixed even within the broader context of shoot versus root.

## **Stem Cell Populations in *Physcomitrella***

Land plants are inferred to have evolved from a group of algae (Timme et al., 2012) in which the meristem consisted of a single stem cell (Graham et al., 2009). The simplex meristem, which contains a single stem cell called an apical cell, is seen in all seedless plants except for two lineages of lycophytes (Lycopodiales and Isoetales) (Schneider et al., 2002), however the properties of these meristems vary greatly depending on the tissue and species. Although the basic body plan of seedless land plants partly resembles that of seed plants, there are remarkable differences in the strategy of organogenesis between complex and simplex meristems. Among plants with simplex meristems, we will focus our discussion to *Physcomitrella*.

In *Physcomitrella*, eight types of cells with stem cell characteristics are formed during the life cycle: chloronema apical cells, caulonema apical cells, gametophore apical cells, leaf apical cells, rhizoid apical cells, antheridium initial cells, archegonium initial cells, and sporophyte apical cells (Figure 4) (Sakakibara et al., 2008; Kofuji et al., 2009).

Protonemata, the filamentous tissue that arises from the *Physcomitrella* spore, consist of two types of cells, the chloronema and caulonema cells, which derive from a chloronema and a caulonema apical cell, respectively. The chloronema apical cell is initiated during spore germination and promotes tip growth of the filamentous body. To produce chloronema cells, the chloronema apical cell continually self-renews and divides along a single plane. Chloronema cells retain some plasticity and are periodically reprogrammed to form secondary chloronema apical cells



**Figure 4. Physcomitrella Stem Cells.** Depiction of the seven types of Physcomitrella stem cells. (A) Chloronema (top) and caulonema (bottom) apical stem cells locating at the tip of chloronema and caulonema filaments, respectively. (B) A gametophore (left) and a drawing of a longitudinal section showing a gametophore apical cell (right, top), and a schematic of a gametophore apical cell with three planes of division (right, bottom). (C) A matured leaf (left) and a drawing of a leaf primordium with a leaf apical cell (right). (D) Rhizoids emerging from a gametophore (top) and a drawing of a rhizoid filament with a rhizoid apical cell at the tip (bottom). (E) A clump of antheridia and archegonia removed from a gametophore apex (left), schematics of developmental stages of an antheridium (middle) and an archegonium (right). Primordium morphology of each gametangium cannot be distinguished. (F) Reprogramming leaf cells to chloronema apical stem cells at the edge of a gametophore leaf after 0, 32, 38, and 44 hours after leaf excision. Stem cells are indicated in black and arrowheads indicate planes of division. Scale bars: 50  $\mu\text{m}$  (A and F); 1 mm (B and D); 100  $\mu\text{m}$  (C and E). Original pictures for drawings in (B), (C) and (E) were provided by Drs. Keiko Sakakibara and Rumiko Kofuji.

that form the branches of the chloronemata. The chloronema apical cell changes into a caulonema apical cell to produce caulonema cells. In turn, the caulonema cells can form three types of cells: secondary chloronema apical cells, caulonema apical cells and gametophore apical cells (Cove and Knight, 1993). Chloronema and caulonema cells can be distinguished by their chloroplast morphology, cell length, tip growth rate, and cross wall orientation (Cove et al., 2006; Prigge and Bezanilla, 2010).

The leafy shoot of *Physcomitrella*, the gametophore, originates from gametophore apical cells within the protonema. Gametophore apical cells are tetrahedral and have three planes of division which generate a stem and leaves (Harrison et al., 2009). Leaf apical cells, originating from the gametophore apical cell, divide along two planes to generate two rows of wedge-shaped cells, thereby generating monolayer leaves. In contrast, rhizoid apical cells initiate from epidermal cells of the gametophore stem and produce brown-pigmented rhizoid cells with undifferentiated chloroplasts through the continuous cell division of a tip cell within a single plane (Sakakibara et al., 2003).

Short-day and cool temperature conditions induce two additional stem cells, the antheridium initial cell and archegonium initial cell, each with two planes of division at the tips of gametophores (Hohe et al., 2002). These initial cells produce several wedge-shaped cells and a series of stereotypic cell divisions form antheridia and archegonia bearing gametes: sperm and an egg (Kofuji et al., 2009). After fertilization, as mentioned before (Figure 2B), the first zygotic cell division forms a sporophyte apical cell with two planes of division, which produces precursor cells of semi-epiphytic sporophytes.

As described above, *Physcomitrella* has multiple types of stem cells with one or more planes of division. In contrast to an apical cell of algae that generates its filamentous body and planar body through division in one or two planes respectively, bryophytes such as *Physcomitrella* have acquired stem cells capable of division in three planes allowing for shoot generation. Apical cells with three or four division planes are also seen in lycophytes and monilophytes and they act as shoot initials through stereotypical cell division patterns (Philipson, 1990; Schneider et al., 2002; Harrison et al., 2007). In mosses, including *Physcomitrella*, spiraling division planes of gametophore apical cells continually generate the spiral phyllotaxis of leaves. Leaf apical cells divide with two planes of division to produce a planar leaf (Harrison et al., 2009). In this way, establishment of multidimensional development is regulated by the arrangement of cross wall orientation in simplex meristem plants. Thus the body plan transition from the simplex meristem is supported by alteration of division planes and well-controlled asymmetric divisions.

## **STEM CELL NICHE IN PLANTS**

In plants, the stem cell niche is morphologically distinct from animal systems, but functions in a similar manner. Within the multiplex meristem, there is a population of cells that is required to maintain a microenvironment conducive for stem cells to remain in an undifferentiated state. In the shoot, this is referred to as the organizing center and in roots, the quiescent center. Although niche cells have not been characterized in simplex meristems,

recent studies in seedless plants indicate that members of gene families known to regulate stem cell niches in angiosperms are also expressed in *Physcomitrella*.

## **The Organizing and Quiescent Centers of Arabidopsis**

Within both the shoot and root meristems, a population of cells exists which is postulated to maintain the stemness of the initial cells. In the shoot this population is known as the organizing center, and in the root, the quiescent center. As discussed above, the organizing center is found underlying the central zone and above the rib zone in the shoot, and the quiescent center is located in the center of the initial cells within the root meristem. The importance of the stem cell niche is highlighted by experiments that either genetically or physically ablated the cells within this region.

The shoot organizing center was first defined by the expression of a transcription factor, *WUS* (*WUSCHEL*) located just below the central zone (Mayer et al., 1998). Mutations in *WUS* result in the failure of the shoot meristem to self-renew or properly specify cell fate (Laux et al., 1996). While the *wus* mutant does not have any apparent defects in root meristem maintenance, a transcription factor of the same family, *WOX5* (*WUSCHEL-RELATED HOMEODOMAIN*), has been revealed to play a similar role for the root quiescent center (Sarkar et al., 2007). The mechanisms by which these transcription factors signal to maintain the stem cell niche will be discussed in a later section.

In contrast to genetic studies, a series of ablation experiments have been undertaken to study the requirement of specific cells within the niche for root meristem maintenance and function. Interestingly, in the root meristem, ablation of all quiescent center cells is not functionally informative; ablation of all cells results in the quiescent center being rapidly replaced by cells of a stele (vascular plus pericycle) origin (van den Berg et al., 1995). In contrast, when just one or two cells from the quiescent center are ablated, the replacement is slower and allows analysis of quiescent center function. With a loss of one or two quiescent center cells, there is a loss of stemness in the initial cells and premature differentiation, but only for initial cells that are in direct contact with the ablated cell (van den Berg et al., 1997). Similar studies have not been feasible in the shoot meristem due to the inaccessibility of the organizing center.

The combination of genetic and ablation studies have cemented the importance of the quiescent center in meristem maintenance. However, it is important to note that recent studies utilizing root tip excision demonstrate that the stem cell niche is only required for the continual growth of the root, and not organogenesis per se, since the tip is able to regenerate without the presence of a meristem (Sena et al., 2009).



The mechanism of niche function in the root and shoot meristems relies on similar factors, however they are not completely analogous. The major difference is in the proliferative behavior of the two niches. In the shoot, the organizing center is continually renewed while in the root the quiescent center rarely undergoes cell division (Weigel and Jürgens, 2002; Laux, 2003).

## **MOLECULAR MECHANISMS OF MERISTEM FORMATION AND STEM CELL MAINTENANCE**

When considering the molecular underpinnings of stem cell maintenance and specification, there are some basic differences in plants versus animals to consider. First, because plants have cell walls, there is no cell movement. Second, intercellular communication is achieved in two ways: i. signaling molecules that move in the spaces between the cell walls, or ii. proteins or mRNAs that move between cells through the plasmodesmata, which act as channels between adjacent cells. Coordinated signals within the meristem are critical for proper timing and directionality of cell division. This section will discuss what is known about the molecular cues regulating stem cell specification and maintenance. Specifically, we will address the role of positional information, hormonal or peptide cues and the transcriptional regulation of these processes in *Arabidopsis* and *Physcomitrella*.

### **Positional Cues Determine Cell Fate in *Arabidopsis***

Since plant cells are immobile, cellular plasticity is important to acquire the correct cell fate for a defined region. Early genetic mosaic (chimera) studies in the shoot meristem revealed that while cell division, elongation and positional cell fate are stereotypical, these are not the primary determinant of cell fate (Reviewed by Poethig, 1989; Szymkowiak and Sussex, 1996). For most plant cells, fate is determined by position rather than lineage, and the direction of cell division is critical to determine a cell's position relative to its neighbors.

Specifically, in the shoot meristem, one of the earliest reports in tobacco leaf demonstrated that when the pattern of cell division is changed to displace a cell into a genetically different layer, that cell will take on the fate of the layer in which it was displaced, and not the layer in which it originated (Stewart and Dermen, 1970). This concept was further solidified by studies with the root meristem. Initially, ablation experiments were undertaken to understand how loss of specific cell types would influence the meristem (van den Berg et al., 1995). Ablation of an initial cell resulted in its replacement by a neighboring cell that subsequently switched fate

to that of the ablated initial cell. These studies demonstrated not only the importance of position, but also the plasticity of cells within the meristem (van den Berg et al., 1995). Subsequent clonal analysis, confirmed the importance of position over lineage in the cell fate decisions of the root (Kidner et al., 2000).

In *Physcomitrella*, the relative importance of lineage and position on cell fate has not been extensively studied. However, when a gametophyte apical cell is ablated in ferns, a neighboring cell becomes the new apical cell (Korn, 1974), indicating that positional information may play a central role in stem cell formation in seedless plants.

While positional information determines cell fate, hormone and peptide signaling are necessary to correctly provide the positional information. Investigations involving hormonal and peptide signaling are numerous, so for the sake of brevity, we will only consider a few examples. Specifically, the importance of hormone and peptide signaling with regard to meristem initiation and maintenance will be discussed.

## **Meristem Initiation in Land Plants**

The signaling mechanisms responsible for the initiation of shoot and root meristems in *Arabidopsis* occur during embryogenesis. Paramount among these signals is the plant hormone auxin. Auxin has been shown to be important for a multitude of processes, thus it is no surprise that it is important in the early stages of axis formation. Specifically, Friml et al. demonstrated that asymmetric auxin localization was observed at the first cell division of the zygote to begin establishing apical-basal polarity in the embryo (Friml et al., 2003). Apical-basal asymmetric distribution of auxin was also observed in the *Physcomitrella* embryo, suggesting a similar mechanism in both organisms (Fujita et al., 2008).

Auxin also plays an important role in the establishment of the *Arabidopsis* root meristem, which forms at the maximal point of auxin concentration (Sabatini et al., 1999). It is not just the amount of auxin present that influences the development of a root meristem, instead, the balance between auxin and a second hormone, cytokinin, is what determines root versus shoot meristem. When plants are regenerated from callus tissue, a high ratio of cytokinin to auxin will result in formation of shoots, while a low ratio will result in roots (Skoog and Miller, 1957). Although cytokinin is critical for the establishment of shoot meristems, its presence has not been detected in the early embryo (Müller and Sheen, 2008). One of the earliest detectable molecular markers of the shoot meristem is expression of a transcription factor, *STM* (*SHOOT MERISTEMLESS*), which is evident at the mid-globular stage of embryogenesis (Long et al., 1996). *STM* has been shown to induce cytokinin biosynthesis thus suggesting it is required

to initiate the cytokinin-dependent specification of the shoot meristem (Jasinski et al., 2005; Yanai et al., 2005). Loss of *STM* results in a loss of the shoot meristem (Long et al., 1996), whereas misexpression of *STM* in the presence of *WUS*, previously described for its importance in organizing center establishment, could induce ectopic organ formation (Gallois et al., 2002). Therefore, *STM* is a key factor for initiation of the shoot meristem.

Auxin and cytokinin are also critical for meristem initiation in *Physcomitrella*. When caulonema cells are reprogrammed to form a new stem cell, the exogenous application of cytokinin enhances the formation of gametophore apical cells (Ashton et al., 1979; Cove et al., 2006). Cytokinin-resistant mutants produce fewer gametophores, however the exogenous application of auxin to these mutants restored gametophore formation (Ashton et al., 1979). Thus auxin, together with cytokinin, positively regulate gametophore apical cell formation (Ashton et al., 1979). However, in contrast to *Arabidopsis*, *STM* orthologs are not necessary for stem cell formation in *Physcomitrella* in the gametophyte or sporophyte generation (Sakakibara et al., 2008).

The *Arabidopsis* root meristem, specifically the quiescent center, forms at the peak of auxin concentration (Sabatini et al., 1999). Auxin has been shown to act through a family of transcription factors called *PLT* (*PLETHORA*) to mediate initiation of the quiescent center (Aida et al., 2004). Aida et al. demonstrated that *PLT1* and *PLT2* are expressed in the basal portion of the embryo responsible for root formation, and act downstream of auxin to define the root meristem. *PLT1* and *PLT2* are highly homologous and proposed to be functionally redundant, however a double knockout of these genes does not prevent root formation, suggesting a role for other proteins as well (Aida et al., 2004). Ectopic expression of the *PLT* genes can alter shoot tissue into root, indicating that *PLT* plays a key role in root meristem initiation (Aida et al., 2004).

*Physcomitrella* has four genes orthologous to *Arabidopsis PLT*. Disruption of all four genes results in plants that are unable to form gametophores indicating that *PLT* orthologs are necessary for gametophore apical cell formation (Aoyama et al., 2012). *Physcomitrella PLT* orthologs are induced by auxin and function in parallel with cytokinin signaling.

Exogenous auxin also stimulates rhizoid development from the gametophore epidermis and transition of chloronema apical cells to caulonema apical cells by positively regulating *RSL* (*RHD SIX-LIKE*) transcription factor genes (Jang and Dolan, 2011; Jang et al., 2011). The *RSL* genes are expressed in cells that give rise to rhizoids and *RSL* overexpression is sufficient for induction of rhizoid apical cells. The orthologous genes in *Arabidopsis* control root hair development, but are not regulated by auxin (Menand et al., 2007; Jang and Dolan, 2011). Although both rhizoid and root

hairs play a role in the uptake of water and nutrients from substratum, the auxin-mediated regulation of *RSL* genes is only seen in *Physcomitrella*.

While these studies represent only a portion of our understanding of the molecular regulation of meristem initiation, they are among the most critical. Taken together, both shoot and root meristem initiation is specified early in the embryo by a combination of hormone and transcriptional cues. In addition, preliminary studies in *Physcomitrella* suggest that at least some of these mechanisms are components of an ancestral genetic regulatory network required for stem cell formation in land plants.

## Meristem Maintenance in Land Plants

Since plants are undergoing continual renewal and generation of adult organs, the maintenance of stem cell populations is as important as their initiation. In particular, we will focus on the signaling pathways between the organizing center and the initial cells that they maintain. As mentioned previously, the organizing center of the *Arabidopsis* shoot is defined by the presence of a transcription factor, *WUS*. Recent work has demonstrated that *WUS* is part of a peptide-signaling feedback pathway. In this pathway, *CLV3* (*CLAVATA3*), a member of the *CLE* (*CLV3/ENDOSPERM SURROUNDING REGION*) peptide family, is expressed in the apical stem cells (above the organizing center), and restricts *WUS* to the organizing center. In a feedback process, *WUS* is also required for the expression of *CLV3* in the apical stem cells (Brand et al., 2000; Schoof et al., 2000). In the absence of *WUS*, the meristem is reduced in size as a result of premature differentiation, whereas in the absence of *CLV3*, the meristem is increased due to an increase in undifferentiated cells (Leyser and Funder, 1992; Clark et al., 1993; 1995). The mechanism of *CLV3* action is proposed to be through binding leucine rich repeat receptors, *CLV1* and *CLV3*, which lie in the rib zone beneath the organizing center, and thus affect the expression of *WUS* (Muller et al., 2008; Ogawa et al., 2008; Matsuzaki et al., 2010). *STM*, as mentioned above is critical for initiation of the shoot meristem, however it also functions in the maintenance of the meristem. Both *STM* and *WUS* are required for the maintenance of initial cells in the shoot meristems (Laux et al., 1996).

A transcription factor similar to *WUS*, *WOX5* is expressed in the quiescent center of the *Arabidopsis* root. Similarly, a member of the *CLE* peptide family, *CLE40*, is expressed in columella cells (distal to the quiescent center and initials), and is proposed to restrict the expression domain of *WOX5* (Sarkar et al., 2007; Stahl et al., 2009). While some parallels have been drawn between the shoot and root meristems with regard to this signal-peptide pathway, the expression domains of the pathway components are not analogous, thus suggesting some important differences (Reddy and Meyerowitz, 2005; Sarkar et al., 2007). The precise signaling cascades

underlying these feedback interactions in both shoot and root are still being parsed out; for a recent review, see Katsir et al. (2011).

In *Physcomitrella*, niche cells have not been recognized, however, a recent report suggests that cells adjacent to an apical cell may function in regulating stem cell properties in fern sporophytes. A *WOX* gene is expressed in the immediate descendants of the root apical cell in the fern *Ceratopteris richardii* (Nardmann and Werr, 2011). Loss- or gain-of-function experiments as well as ablation of these *WOX*-positive cells could provide insight as to whether the *C. richardii* *WOX* gene functions in maintenance of stem cells. By contrast, in *Physcomitrella*, protonema apical cells appear to function without niche cells. When a cell adjacent to a protonema apical cell is ablated or a protonema apical cell is isolated, the apical cell continues to divide and grow to produce protonema cells. The *Physcomitrella* genome lacks an ortholog to *CLV1*, although there are several closely related homologs, which could substitute for *CLV1* function (Banks et al., 2011). However, *Physcomitrella* *WOX* orthologs are ubiquitously expressed in both gametophyte and sporophyte generations and do not function in the maintenance of stem cells (Sakakibara et al., in submission). Thus, as yet there is no evidence for the existence of supporting niche cells in *Physcomitrella* and even if such evidence arises, it is likely that stem cells are maintained through different mechanisms from angiosperms.

In the *Arabidopsis* root, two additional pathways are critical for the maintenance of stem cell populations, the *SHR/SCR* and *PLT* transcription factors. *SHR* and *SCR* are transcription factors of the plant-specific GRAS family that are required for quiescent center identity, as well as radial patterning (Di Laurenzio et al., 1996; Helariutta et al., 2000; Wysocka-Diller et al., 2000; Sabatini, 2003). *SHR* is transcribed in the vascular tissue and the protein moves outward into the quiescent center, cortex/endodermal initials and the endodermis to initiate the transcription of *SCR* (Nakajima et al., 2001). Loss of *SCR* or *SHR* results in aberrant quiescent center cells and a cessation of root growth; these defects are attributed to the requirement of *SCR* and *SHR* for the maintenance of the root supporting niche cells (Scheres et al., 1995; Di Laurenzio et al., 1996; Sabatini, 2003). GRAS genes including *SCR* and *SHR* orthologs are found in the *Physcomitrella* genome but their function is still unknown.

As mentioned above, *PLT* transcription factors are important for the initiation of the root meristem, but they are also important for its maintenance. Double knockout of *PLT1* and *PLT2* results in a small root meristem, with fewer initial cells that rapidly differentiate after germination. Thus, in addition to the role of *PLT* genes in meristem initiation, they are also required for meristem maintenance (Aida et al., 2004). *PLT* genes act in parallel with the *SHR/SCR* pathway to define the quiescent center and maintain stem cell potential within the root (Aida et al., 2004). In

Physcomitrella, *PLT* orthologs function in the initiation of gametophore stem cells but not in their maintenance, as they are not expressed at the gametophore apex (Aoyama et al., 2012).

## Molecular Mechanisms of Cell Fate Determination

The first step in the development of any organ is the initiation and maintenance of the stem cell population, however other cellular transitions are required to produce a differentiated and functional tissue. After a stem cell undergoes asymmetric division, the daughter cell must receive the proper cues to expand as a transit amplifying population, elongate and differentiate to a terminal cell fate. In the Arabidopsis root this process occurs within the meristematic, elongation and differentiation zones respectively. The current state of understanding of these processes in Arabidopsis is described in this section.

The bi-potential cell fate decision that is critical for some initial cells has been studied from various perspectives in Arabidopsis. Most studies focus on root initial cell fate determination because of the radial patterning and simple architecture. Much of our knowledge about the specification of initial cells comes from work on the cortex/endodermal initial. Specifically, the aforementioned *SHR* and *SCR* pathway was originally characterized through its role in the specification of cortex and endodermal cell fates (Benfey et al., 1993; Scheres et al., 1995). *SHR* is transcribed in the stele and *SHR* protein moves into the CEI daughter and endodermis to regulate endodermal cell fate (Nakajima et al., 2001; Cui et al., 2007). In a *shr* mutant, there is a single layer of cells with cortex identity (Benfey et al., 1993; Di Laurenzio et al., 1996; Helariutta et al., 2000).

Another well-studied cell fate decision is the partitioning of the epidermis into hair and non-hair cell identity. The epidermis overlies the cortex, and cell positioning relative to the cortex determines cell identity. Specifically, hair cells are specified from epidermal cells that overlie cortex cell junctions, and non-hair cells are specified from epidermal cells that are in contact with a single underlying cortex cell (Dolan et al., 1994; Galway et al., 1994). A leucine-rich repeat receptor-like kinase, *SCM* (*SCRAMBLED*), is proposed to mediate this positional information (Kwak, 2005). *SCM* utilizes a feedback loop with downstream transcription factors to reinforce its expression in hair cells and promote hair cell fate while repressing non-hair cell fate (Kwak and Schiefelbein, 2008). In addition to the studies highlighted here, there are numerous cell fate specification studies in other cell types within the shoot and root.

In the Arabidopsis root, after a cell exits the highly proliferative meristematic zone, it enters the elongation zone where it expands and initiates programs to terminally differentiate. The molecular cues that

regulate the transition from transit amplifying cell to differentiated cell remain largely undiscovered. However, a recent study identified a transcription factor, *UPB1*, which regulates the transition between cell proliferation and differentiation (Tsukagoshi et al., 2010). *UPB1* was shown to modulate the balance of reactive oxygen species at the transition zone (where the meristematic zone becomes the elongation zone), and control the onset of differentiation (Tsukagoshi et al., 2010). Thus we are just beginning to understand the molecular cues that regulate terminal differentiation in Arabidopsis.

## Regeneration and *de novo* Formation of Stem Cells

An exciting aspect of stem cell research is the ability of stem cells to mediate regeneration, as well as the prospect of *de novo* formation of stem cells from differentiated cells. The plasticity inherent to plant cells makes them an exceptional system in which to study these processes. Highlights of these studies in both Arabidopsis and Physcomitrella are presented here.

## Regeneration in Plants

Regeneration has been studied from two points of view in plants. The first, a classical approach, is to excise a portion of the plant and observe the properties of regeneration as it replaces that tissue. The second is plant-specific, and concerns the generation of pluripotent stem cells with or without a stage of callus. Both approaches will be discussed here.

For classic regeneration experiments, Arabidopsis root and leaf excision are most well studied. While ablation experiments in the Arabidopsis root tip, as described earlier, have demonstrated the ability of regeneration at the cellular level, one group studied the ability of the entire root tip to regenerate in the absence of a meristem (Sena et al., 2009). In this study, the authors excised part of the meristematic zone, above the quiescent center (Figure 3B), and the root successfully regenerated. Although no specific tissue or cell type was solely responsible for the regeneration, the presence of some cells in the meristematic zone was required for regeneration (Sena et al., 2009). Further, in *plt1/plt2* double mutant plants and *scr* mutant plants, in which premature differentiation of root initials results in stunted roots, regeneration still occurred to reestablish the pre-excision morphology (Sena et al., 2009). Thus, the signaling mechanisms required for organogenesis are distinct from those required for post-embryonic growth and elongation.

Loewenberg described one of the first studies of callus formation in Arabidopsis in 1965 (Loewenberg, 1965). By definition, calli are masses of tissue from which an entire plant can be regenerated. The generation

of callus as a means of whole plant regeneration can be achieved in two ways, either by wounding the plant and inducing formation of callus at the wound site, or by explanting cells into tissue culture. Most often callus generation is achieved by root explants, as roots are extremely amenable to this type of transformation.

Interestingly, regardless of the source tissue, calli express markers from the root meristem, suggesting that callus formation is not due to a process of de-differentiation and re-differentiation, but instead is an inherent and reproducible mechanism of induced pluripotency (Sugimoto et al., 2010). This inherent mechanism may be the nature of how the callus is formed. For example, to induce shoot regeneration from calli, the tissue first enters an auxin-induced callus-phase, followed by a low auxin, high cytokinin shoot induction phase (Skoog and Miller, 1957; Che et al., 2007). In the absence of this auxin-induction, a wide array of genes, including *WUS*, are not induced (Che et al., 2007). Thus, the callus itself may have properties resembling the root meristem due to the auxin-induction. Importantly, during shoot induction from callus, genes important for the initiation of the shoot meristem, such as *SMT* and *WUS* come on in the correct spatial and temporal order (Cary et al., 2002; Gordon et al., 2007).

While it is often believed that all plant cells are totipotent, recent evidence contradicts this dogma. Indeed, multiple studies have demonstrated that callus tissue tends to arise from cells of the meristem and pericycle founder cells (Che et al., 2007; Gordon et al., 2007; Atta et al., 2009), the differentiation potential of which has been understood for lateral root initiation as discussed below. In further support of this idea, a recent study demonstrated plants that are unable to initiate lateral roots could also not initiate callus formation (Sugimoto et al., 2010). Classic experiments in leaf regeneration have suggested that age is also a factor in regenerative capacity. Sena et al. provided further support for this hypothesis when they showed that older leaves have a reduced ability to regenerate as compared to younger leaves (Sena et al., 2009). Together these studies suggest that while all plant cells may have plasticity, current evidence for the mechanisms of regeneration is attributable to either known stem cell populations or generally immature tissue.

### ***De novo* Formation of Stem Cells in Arabidopsis: Lateral Root Induction**

One of the unique properties of root development is the generation of lateral roots *de novo* from differentiated root tissue. Developmentally, lateral root formation is not associated with the root meristem and occurs in more mature or differentiated tissue. In support of this idea, plants



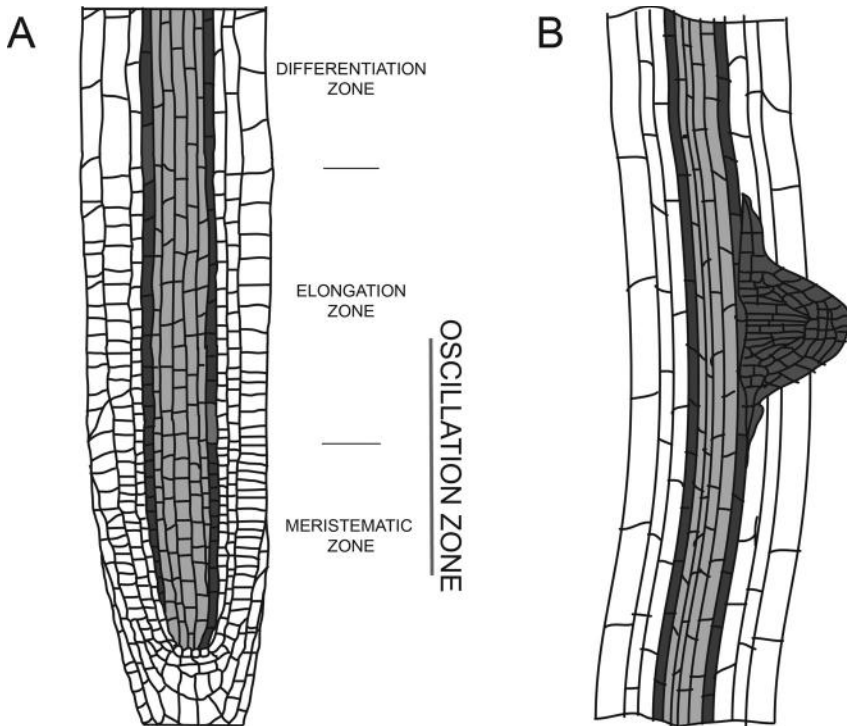
mutant for transcription factors responsible for root meristem initiation and maintenance, *shr*, *scr*, or *plt1/plt2*, have lateral root emergence, but with the same radial patterning defects as observed in the parent root (Di Laurenzio et al., 1996; Aida et al., 2004; Lucas et al., 2011).

Interestingly, the founder cells for lateral root initiation are found in the stele, specifically the xylem pole pericycle cells (Dubrovsky et al., 2000; Beeckman et al., 2001; Kurup et al., 2005). As mentioned above, pericycle cells have been found to contribute to regeneration during callus formation (Che et al., 2007; Gordon et al., 2007; Atta et al., 2009), suggesting that pericycle cells maintain some stem cell potential in the adult organ. Indeed, mutants that have only one xylem pole, and thus unilateral xylem pole pericycle cells, display consequent unilateral lateral root formation (Parizot et al., 2008). However, a mutant where all pericycle cells have a xylem pole association also displays reduced lateral roots, suggesting that intercellular signaling between xylem pole and non-xylem pole pericycle cells is also critical for proper lateral root initiation (Parizot et al., 2008). The nature of this intercellular communication remains elusive at this time.

While the cellular origin of lateral roots has been determined to be the xylem pole pericycle cell, not all xylem pole pericycle cells give rise to a lateral root. These cells are competent to form lateral roots, but require external signals to initiate the process. The mechanisms underlying lateral root initiation and positioning have been the source of extensive study and much debate. Originally, studies using a synthetic auxin-responsive reporter suggested that, much like root meristem specification, lateral roots formed at a fluctuating maximum of auxin signaling (De Smet et al., 2007). However, a recent study demonstrated that while the synthetic reporter is oscillating coincident with lateral root specification, it is not directly in response to auxin. Instead, an entire network of genes (approximately 3,400) is oscillating within an oscillation zone (late meristematic/early elongation zone) to position lateral roots (Figure 5A) (Moreno-Risueno et al., 2010). Thus an inherent time-keeping mechanism is responsible for the periodic positioning of lateral root founder cells.

Subsequent to the specification and positioning of lateral root founder cells, lateral root initiation occurs. In contrast to specification and positioning, lateral root initiation is auxin-dependent (Dubrovsky et al., 2008). Auxin is proposed to induce nuclear migration towards a shared wall in adjacent lateral root founder cells (De Rybel et al., 2010). The significance of this nuclear migration is not well understood, but if migration occurs in only one of the cells lateral root initiation is inhibited (De Rybel et al., 2010).

Following lateral root initiation, the lateral root founder cells undergo asymmetric division within the differentiation zone, a region of low mitotic index, to initiate the formation of a lateral root primordium (Figure 5B). A series of stereotypical cell divisions expand the lateral root primordium



**Figure 5. Lateral Root Formation in Arabidopsis.** (A) Root diagram depicting the specification of lateral roots in Arabidopsis. Light brown indicates the stele (vascular) tissue. Dark brown indicates the pericycle cells of the root. Lateral root position is defined by an inherent time keeping mechanism of gene oscillation within an oscillation zone. Red indicates the future site of lateral root formation. (B) Lateral root emergence occurs within the differentiation zone through a series of stereotypical divisions. After emergence from the parent root, the newly formed lateral root meristem is responsible for root growth.

*Color image of this figure appears in the color plate section at the end of the book.*

to penetrate the surrounding endodermal layer. Subsequent cell divisions result in a structure resembling the root meristem and lateral root emergence from the parent root. After lateral root emergence, cell division originates in the lateral root meristem and initial cells become the mechanism by which lateral roots grow and develop (Malamy and Benfey, 1997).

### **De novo Formation of Stem Cells in Physcomitrella**

*Physcomitrella* cells also have a high capacity for regenerative plasticity (Cove and Knight, 1993). When gametophore leaves are excised, leaf cells facing the cut undergo tip growth similar to that of a chloronema apical cell, which is interpreted as leaf cells being reprogrammed to chloronema

apical cells (Ishikawa et al., 2011). Furthermore, this process does not require the addition of any exogenous hormones, but instead is suggested to use intrinsic auxin and cytokinin signaling pathways (Nishiyama et al., 2012). In contrast to *Arabidopsis*, this cellular transition does not mediate the formation of callus. Since the majority of the leaf blade of *Physcomitrella* is composed of a single cell layer, it is easy to observe the changing cellular morphology and to determine which cell is being reprogrammed. Because of these advantages and the ease of manipulation, the leaf-cut assay has recently emerged as a preferred experimental system for the study of *de novo* stem cell initiation in *Physcomitrella*.

Using the leaf-cut assay, Ishikawa et al. identified the cell cycle regulator *CDKA;1* (*CYCLIN-DEPENDENT KINASE A;1*) as a coordinator of the rapid change of cell identity and cell cycle progression (Ishikawa et al., 2011). A DNA synthesis inhibitor, aphidicolin, inhibited only cell division, but prevented neither tip growth nor chloronema gene expression. This result indicates that leaf-cell reprogramming can be separated into the acquisition of stem cell characteristics and cell cycle progression. On the other hand, a CDK inhibitor or dominant-negative *CDKA;1* inhibited not only cell division but also tip growth and chloronema gene expression, indicating that *CDKA;1* links cell cycle progression with acquisition of cell identity characteristic to the chloronema cell during reprogramming. Although the *Physcomitrella* reprogramming system has provided a clear example of coordination of reprogramming, the underlying molecular network remains unclear. Future studies will be directed towards the identification of key regulators participating in the reprogramming and are expected to uncover the general and specific molecular mechanisms controlling stem cell initiation in plants.

## **Stem Cells in Other Plant Systems**

While this chapter has focused on the regulation, specification and regeneration of stem cells in *Arabidopsis* and *Physcomitrella*, the mechanisms described here are applicable across many plant systems. One major difference is the shoot meristem organization between angiosperm, as described here via *Arabidopsis*, and gymnosperm. Whereas angiosperms have three layers (L1, L2, and L3), gymnosperms have two layers, one that is equivalent to the L1 and L2 layers, and the other is comparable to the L3. Despite this difference, common regulatory mechanisms, conserved from an ancestor of land plants, are found among a multitude of plants.

## SUMMARY AND CONCLUSIONS

The convergent evolution of stem cells in plants and animals provides an interesting perspective from which stem cell regulation can be studied. The similarities and differences between these two populations help to deepen our understanding of general stem cell regulation. Similarities such as hormonal (in plants) or growth factor (in animals) gradients to determine the site of stem cell initiation, allow us to infer general mechanisms that are critical for stem cell formation regardless of evolutionary origin. Other similarities include the amount and distribution of reactive oxygen species, the requirement for nuclear migration in some stem cell populations and the requirement of stem cell maintenance signals whether from a supporting cellular niche or elsewhere. Interestingly, the study of *Physcomitrella* with a stem cell, but as yet no identified niche, redefines our view of the need for supporting cells. Further research into the cues necessary to maintain this stem cell will advance our understanding of the role of the niche.

Another aspect of stem cell regulation is the *de novo* formation of new stem cell populations resulting in organ regeneration. While some organs within mammals are continually regenerating by utilizing a stem cell population (i.e., skin, intestine and hematopoietic cells), the majority of organs are unable to regenerate. In contrast, plants are highly regenerative at the cellular, tissue and whole plant level. Whether this difference in regenerative capacity is due to the maintenance of stem populations in adult plants, the reduced complexity of the plant body plan or an inherent difference in plants and animals remains to be determined.

In this chapter, we have highlighted the hallmarks of stem cell formation and maintenance in land plants. Specifically we have focused on *Arabidopsis* and *Physcomitrella* as representative seed and seedless plants and have attempted to highlight examples that demonstrate how plants provide a tractable experimental system for the study of stem cell regulation.

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## ABBREVIATION

CEI cortex/endodermis initial

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# Planarian Totipotent Stem Cells

*Teresa Adell, Francesc Cebrià and Emili Saló\**

## SUMMARY

Planarians (freshwater flatworms) are able to regenerate a complete animal, including the brain and reproductive system, after any kind of amputation. Although their striking regenerative capacity has attracted generations of biologists, recent years have seen them transformed into an essential model for the study of regeneration and stem cell biology using modern molecular and genomic tools. The impressive plasticity of planarians is based upon the presence in adult animals of a unique type of totipotent stem cells named neoblasts. In this chapter, we review current understanding of the molecular mechanisms that direct body patterning and control neoblast maintenance and differentiation in planarians. We provide an overview of the large volume of data obtained mainly in the last 5 years on the molecular mechanisms underlying the establishment and maintenance of axial polarity by the Wnt and BMP signalling pathways. We also review recent approaches to elucidate the mechanism of neoblast differentiation into specific cell types and organs.

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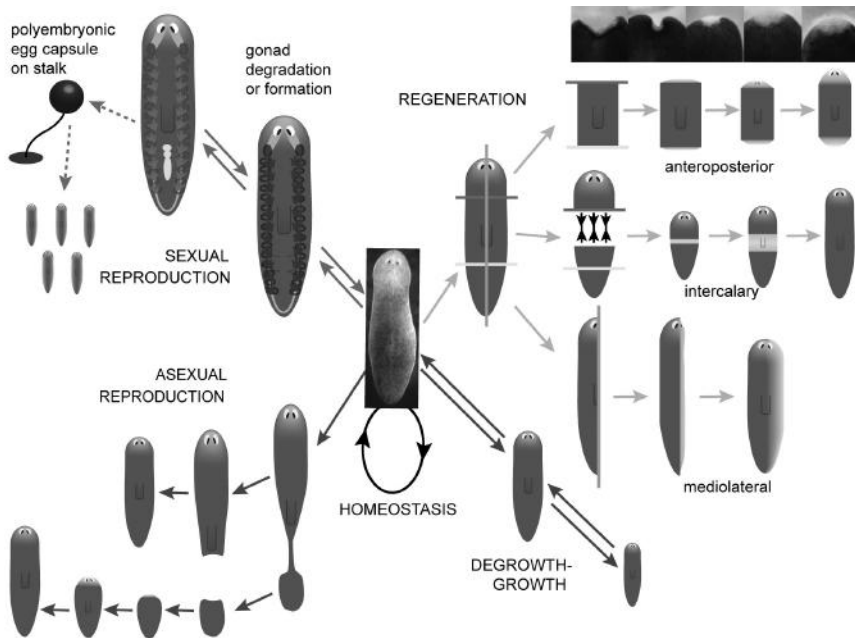
*List of abbreviations after the text.*

## INTRODUCTION

Planarians (*Platyhelminthes*, *Tricladida*) are triploblastic acoelomates that are flattened along the dorsoventral axis (explaining why they are commonly referred to as flatworms) to generate a bilaterally symmetrical body plan. These animals are mainly known because of their striking regenerative abilities (Brøndsted, 1969; Reddien and Alvarado, 2004; Saló, 2006). Thus, if a single planarian is chopped into several pieces, each of these fragments will regenerate a whole animal in just a few days (Figure 1). The most frequently used flatworms in regeneration studies are free living freshwater planarians, usually a few millimetres long. In addition to regenerating completely after any kind of amputation, they exhibit a continuous remodelling activity in which they grow or degrow according to the availability of food (Figure 1).

The epidermis of planarians consists of a monolayer of ciliated epithelial cells with a higher density of cilia on the ventral side of the animal. These cilia are responsible for locomotion along a mucous trail produced by a large number of secretory cells located under the ventral basal lamina. Planarians are among the largest animals in which cilia are used to control movement, although under stress conditions they use muscle contraction to move faster. Beneath the basal lamina, there are four layers of body wall musculature orientated in different directions (Cebrià et al., 1997). The planarian nervous system is composed of two ventral nerve cords (VNCs) that extend along the length of the organism. At the anterior end, two bilateral cephalic ganglia lie dorsally to the VNCs and connect to sensory and chemosensory cells in the head periphery. A pair of eyes, located dorsally to cephalic ganglia are responsible for sensing light. Planarians lack a skeletal system, but are turgid. Although they also lack a circulatory system, oxygen diffuses directly into the tissues from the surrounding medium. The digestive system is blind, as the animals lack an anus. It is composed of a pharynx, located in the middle of the flatworm that connects to a highly branched gut, with three main branches: one anterior and two posterior. The pharynx is a highly innervated and motile organ that evaginates through the ventral mouth opening to ingest the food.

There are two modes of reproduction in planarians: asexual, by fission, and sexual, involving pair-wise mating (they are hermaphrodites with cross-fertilization) (Figure 1). Some species combine both modalities according to the environmental conditions, while others utilize only a single mode of reproduction. Despite being Lophotrochozoans, planarians have lost the typical spiral cleavage, and their embryonic development is highly modified (Cardona et al., 2005). A single cocoon (egg capsule) contains



**Figure 1. Planarian homeostasis and regeneration. Growth–degrowth.** Planarians are very plastic, and upon starvation there is a reduction in the total body cell number (degrowth) caused by an increase in the rate of cell death compared with that of cell proliferation. After feeding, the planarian will grow in size and the cell number will increase again (growth) through a shift in the equilibrium to favour mitosis over cell death. **Regeneration.** Practically any imaginable amputation of the planarian can give rise to two individual animals. Following transverse cuts, the middle fragment forms both an anterior and a posterior blastema. The head, including the eyes and the brain, is regenerated in the anterior blastema and the tail in the posterior blastema. A process of remodelling takes place in the middle fragment, adjusting the organs to the new smaller size of the planarian. When two fragments with different positional values are joined together, intercalary regeneration reforms the missing tissues between the two regions and gives rise to a single planarian. Finally, a sagittal cut induces the formation of a lateral non-pigmented blastema along the length of the planarian body. The missing eye differentiates and the remaining organs are remodelled, giving rise to an entirely regenerated planarian that is smaller than the original animal. **Reproduction.** Some planarian species can alternate between asexual and sexual reproduction depending on factors such as temperature and nutrient availability. Asexual reproduction takes place by fission, usually in the posterior part of the planarian. Sexual reproduction takes place by cross-fertilization between two hermaphroditic planarians producing a polyembryonic egg capsule termed a cocoon. Hatching occurs after 10 days to several weeks depending on the planarian species. **Homeostasis.** A continuous process of renewal of all planarian cells occurs. Modified from Handberg-Thorsager et al., 2008.

*Color image of this figure appears in the color plate section at the end of the book.*

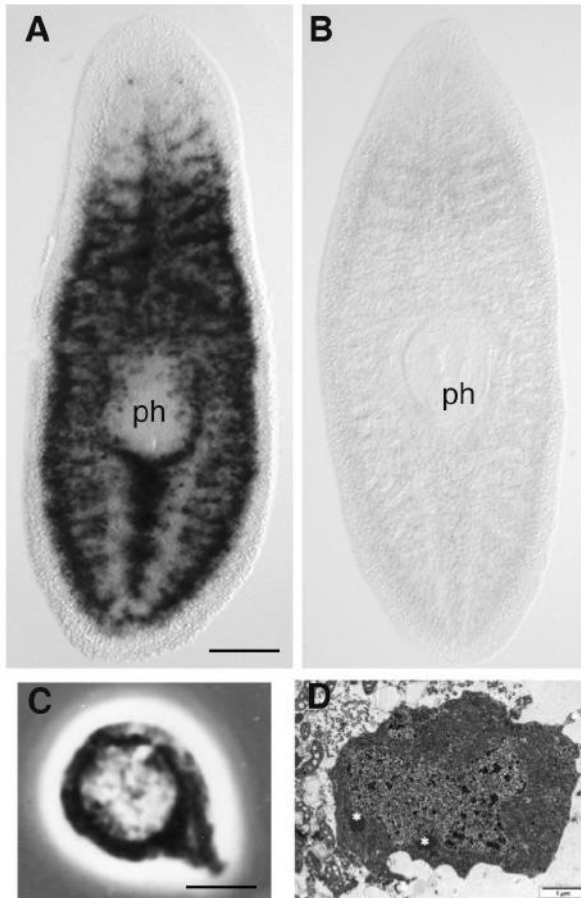
between 5 and 20 eggs (depending on the species) surrounded by a large number of yolk cells. During cleavage, blastomeres (embryonic cells) show an anarchic behaviour, as they remain completely isolated one from the other and migrate towards the periphery of the embryo. Some blastomeres organize themselves into an embryonic pharynx that ingests the yolk cells. Once the embryo is fed, blastomeres start to migrate towards more internal positions and differentiate to form the primordial tissues and organs. At the same time, the embryo elongates and flattens, assuming the definitive axial polarity. After two weeks, small juveniles come out from the cocoon.

In recent years, successful application of a variety of cellular and molecular biological tools has led to the re-emergence of planarians as a model system for research into regeneration. The genome of the *Schmidtea mediterranea* sexual strain has been sequenced by whole-genome shotgun sequencing (Robb et al. 2008; [http://genome.wustl.edu/genomes/view/schmidtea\\_mediterranea/](http://genome.wustl.edu/genomes/view/schmidtea_mediterranea/)), and several ESTs and transcriptome databases are available (Zayas et al., 2005; Abril et al., 2010; Blythe et al., 2010; Sandmann et al., 2011). In addition, gene expression can be analyzed by *in situ* hybridisation (Umesono et al., 1997) and functional analyses can be carried out by RNA interference (RNAi) to silence individual genes (Sánchez Alvarado and Newmark, 1999). Cellular methods such as fluorescence-activated cell sorting (FACS) have also been used to isolate and analyze neoblasts and differentiated planarian cell types (Asami et al., 2002; Hayashi et al., 2006). This combination of tools has led to significant advances in our understanding of regeneration and homeostatic maintenance in planarians.

In this review, we will describe what is currently known about planarian stem cells and their regulation before discussing the molecular mechanisms underlying the establishment of axial polarity in these animals. Finally, we discuss current understanding of the process of cell differentiation and organogenesis in planarians.

## **NEOBLASTS: PLANARIAN TOTIPOTENT STEM CELLS**

The quite limitless regenerative capacity of freshwater planarians relies upon the presence of a unique type of stem cells called neoblasts. Neoblasts are the only mitotically active cells in the adult planarian. They are distributed throughout the parenchyma, the space between the structured organs, with the exception of the pharynx and the most anterior region of the head (Figure 2). Neoblasts account for 20%–30% of the total cell number in adult planarians, depending on the adult size; large animals have a smaller percentage whereas small planarians have a greater proportion of neoblasts, with a maximum of 35% in organisms measuring 3mm in length (Baguña, 2012).



**Figure 2. Planarian stem cells or neoblasts.** (A) Whole mount *in situ* hybridization for the neoblast marker *Smedwi-1* reveals the widespread distribution of neoblasts throughout the parenchyma, except in the head tip and pharynx. (B) After X-ray irradiation, the expression of *Smedwi-1* disappears. (C) Dark-field image of an isolated neoblast revealing the characteristic comma shape. (D) Electron microscopy view of an undifferentiated neoblast showing the low volume of cytoplasm and the chromatoid bodies (asterisks). ph: pharynx. Scale bars: A-B, 0.5 mm; C, 5  $\mu$ m; D, 1  $\mu$ m.

When a planarian is cut, the wound is immediately closed by muscle contraction and then rapidly covered by a thin layer of epidermis that seals the edges of the cut. Fifteen to thirty minutes later, the muscle relaxes and the epidermis stretches to produce a thin layer that covers the wound. An initial generalized mitotic response of the neoblasts (after 6 h) is followed by a second and more local wave of mitosis (after 48 h) below the wound (Saló and Baguña, 1994; Wenemoser and Reddien, 2010). As a consequence, a mass

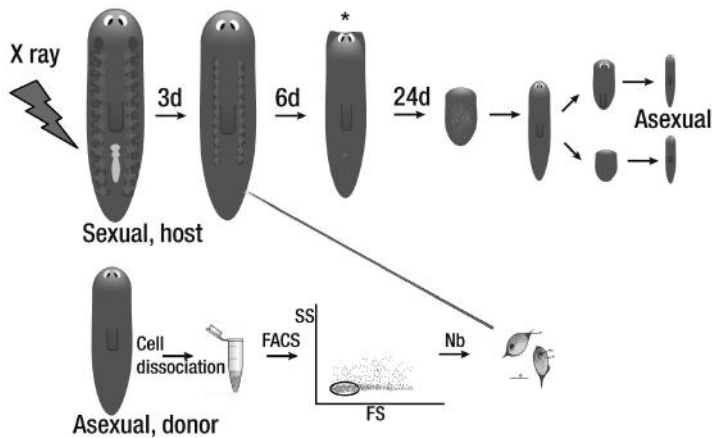
of new undifferentiated tissue called the regenerative blastema grows in the wound area. Within the blastema, the neoblasts differentiate to replace the lost tissues. At the same time, the body re-adjusts to the new proportions of the regenerated planarian. In two weeks, a complete, proportioned, and functional organism is regenerated (Figure 1).

What is the origin of the term 'neoblasts'? In 1891, Harriet Randolph reported that some species of earthworms contain large, undifferentiated, embryonic-like cells with a high nuclei-to-cytoplasm ratio, and she named them neoblasts (Randolph, 1891). Later on, she applied the term to similar, although smaller, cells in planarians (Randolph, 1897). Under the optical microscope, the planarian parenchyma appears as an intermingled mixture of cell types in which it is difficult to define the cell limits, and this led to erroneous suggestions that syncytial structures exist in the planarian parenchyma. In the 20th century, the use of electron microscopy allowed to define more precisely the cellular morphology of neoblasts. Thus, neoblasts were defined as small cells (7–12 microns of diameter) with a typical spindle shape due to the presence of a single filopodia, a large ovoid nucleus with several nucleoli and a thin cytoplasm which results in a high nucleocytoplasmic ratio (Figure 2).

What is the nature of the cells that build the lost parts during planarian regeneration? This question has been challenging biologists for more than 100 years. In the mid-20th century, experiments by Dubois and Wolf provided an important indication of the basic role of neoblasts in blastema production (reviewed in Brøndsted, 1969). This work was based on the observation that killing the neoblasts with ionizing irradiation eliminates the capacity of planarians to regenerate and maintain cell turn-over, and results in non-regenerative flatworms that die several weeks after irradiation. By shielding various portions of the body from irradiation and then examining the regenerative capacity of irradiated organisms, it was shown that the length of time required for regeneration is proportional to the area of irradiated tissue between the wound and the non-irradiated regions (Dubois, 1949). These results were interpreted as reflecting the migration of neoblasts from the non-irradiated tissue to the wounded region, where they proliferate and give rise to the new blastema. Subsequent experiments by Saló and Baguña (1985), using nuclear and cytoplasmic markers together with grafting techniques to follow the movement of neoblasts and differentiated cells, showed that this migration is partially related to the spreading associated with mitotic activity. A second big question is related to the origin of neoblasts: are they derived from a dedifferentiation processes or is there a stable population of stem cells in adult planarians? To try to answer that question, a mosaic strain of *Schmidtea polychroa* in which somatic cells are triploid and premeiotic germline cells are either hexaploid (female germline) or diploid (male germline) was used.

Karyological and cytophotometric analyses revealed that after three days of regeneration around 5% of the nuclei within the blastema contained a diploid chromosome complement (Gremigni et al., 1980), suggesting that premeiotic germ cells may contribute to the formation of blastema cells. However, an alternative explanation could be that this contribution is due to a switch in the state of determination of the germ cells rather than a bona fide transdifferentiation event. A few years later, Baguña et al. (1989) provided strong evidence that neoblasts were really totipotent stem cells, with no evidence of dedifferentiation phenomena. Taking advantage of size differences between neoblasts and differentiated cells of the planarian, serial filtration and density-gradient centrifugation were used to prepare live cell fractions enriched in either neoblasts or differentiated cells. These fractions were then introduced into hosts previously irradiated with a lethal dose of X-rays. Only when the irradiated host was injected with the fraction of enriched neoblasts could regenerative abilities and long-term survival be restored. In contrast, injection of the differentiated cell fraction into irradiated planaria did not rescue mitotic activity, regeneration, or survival. Furthermore, transplantation experiments involving sexual and asexual races of *S. mediterranea* provided compelling evidence that neoblasts serve as stem cells for all differentiated cell types of the planarian, including the germline. When neoblasts isolated from the sexual race were injected into irradiated hosts from the asexual race, those asexual individuals developed germ cells and copulatory apparatus, mated, and laid cocoons. Conversely, when neoblasts from the asexual race were injected into an irradiated sexual host, formerly sexual individuals reproduced instead by fission. Thus, neoblasts are capable of transforming one planarian race into another, strongly suggesting that neoblasts can give rise to all differentiated cell types of the organism. Twenty-two years later, Peter Reddien's lab reported the final answer to the question of whether neoblasts present in adult planarians maintain their pluripotency (Wagner et al., 2011). They used lethal irradiated hosts that were transplanted with single neoblasts isolated by flow cytometry. In some cases, but not always, injection of a single neoblast resulted in the formation of large descendant-cell colonies *in vivo* that later gave rise to several differentiated cell types, restoring body homeostasis and regenerative capacity (Figure 3). From those experiments it can be concluded that neoblasts are a mixed population of cells with different proliferation and differentiation potential and that include a population of totipotent stem cells named cNeoblasts (clonal neoblasts, Wagner et al., 2011). In summary, in an intact planarian a probable mixture of totipotent neoblasts and neoblasts in different stages of commitment drive tissue maintenance through all differentiated cell types.





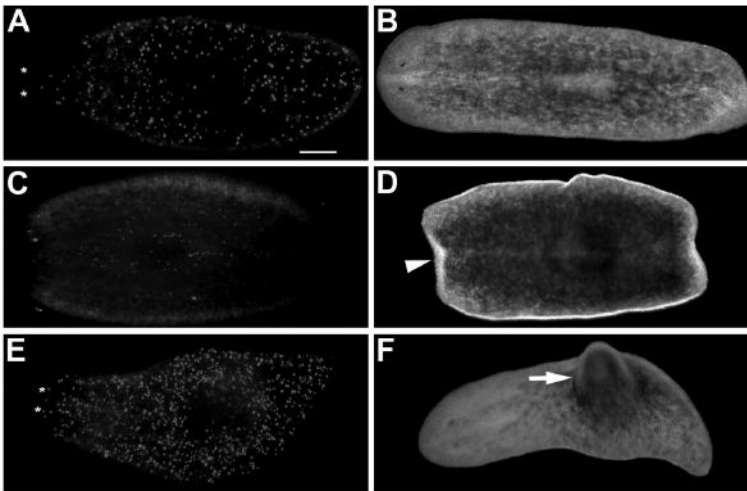
**Figure 3. Neoblasts are totipotent stem cells.** Diagram of the procedure employed to introduce a single neoblast (Nb) from non-irradiated asexual donors into the posterior parenchyma of an irradiated sexual host (based on Wagner et al., 2011). The survivors recover all the regenerative capacities of an asexual planarian.

*Color image of this figure appears in the color plate section at the end of the book.*

## Regulation of Neoblast Maintenance, Proliferation, and Differentiation

Despite those recent experiments showing the totipotency of neoblasts (Wagner et al., 2011), the capacity to label them with BrdU (Newmark and Sánchez Alvarado, 2000), their ultrastructural heterogeneity (Higuchi et al., 2007), and the possibility to follow some cell lineages (Eisenhoffer et al., 2008), there are still many basic questions that remain to be satisfactorily resolved. For instance, what is the percentage of real totipotent neoblasts? Are there committed precursors for every cell type? Are different types of neoblasts associated to different tissues? What are the exact mechanisms that control neoblast proliferation and differentiation? How do the neoblasts precisely respond to amputation or wounding? Some of these questions are now beginning to be answered through RNAi-based functional analyses.

As with any stem cell, neoblast proliferation needs to be tightly regulated. Thus, either blocking the capacity of neoblasts to proliferate or depleting their population leads to the loss of regenerative capacity (Figure 4). In contrast, an excess of neoblast proliferation can lead to the formation of overgrowths or tumors (Figure 4). Deciphering how this regulation is achieved is essential in order to understand not only the cellular basis of planarian regeneration but also the role of stem cells in processes such as tumorigenesis. It has been suggested, for instance, that



**Figure 4. Effects of the misregulation of neoblast proliferation.** (A, C, E) Immunostaining with anti-phospho-histone H3, a marker of mitotic cells. (B, D, F) Live planarians. Compared to a control situation (A, B), inhibition of neoblast proliferation (C) usually correlates with the inability to form a blastema and regenerate (D). Arrowhead points to a practically absent blastema after 7 days of regeneration. In contrast, abnormal neoblast hyperproliferation (E) can lead to the development of ectopic outgrowths (arrow in F). In all panels anterior is to the left. Asterisks mark the photoreceptors.

animals with high regenerative capabilities (such as planarians) appear to be more refractory to the development of spontaneous or induced tumors (Oviedo and Beane, 2009).

In recent years, several genes that appear to play a role in the maintenance of the neoblasts have been identified (reviewed in Aboobaker 2011; Gentile et al., 2011). Thus, the silencing of *Smed-bruli*, an RNA-binding protein, allows the early stages of regeneration to take place normally, as neoblasts initially respond to the amputation by building a blastema and differentiating within it (Guo et al., 2006). After a few days, however, those blastemas stop growing, proliferation rapidly decreases, tissue regression is observed, and finally the animals die. These results are consistent with *Smed-bruli* having a role in the maintenance of the neoblasts by regulating their self-renewal (Guo et al., 2006). Similar roles in neoblast self-renewal and maintenance have been suggested for other RNA-binding proteins such as *Djpum*, a *pumilio* homologue (Salveti et al., 2005), *Spoltud-1*, a Tudor domain-containing gene (Solana et al., 2009), and *Smed-SmB*, a planarian member of the LSm family of RNA-binding proteins (Fernández-Taboada et al., 2010). Another gene involved in neoblast maintenance is *Smedinx-11*, an innexin gap junction channel gene that has been shown by gene silencing to be required for blastema formation and regeneration (Oviedo and Levin, 2007). There are some interesting differences, however, between

the silencing of genes such as *Smed-bruli* or *Spoltud-1*, where the neoblasts appear to respond normally to wound signaling by initially forming normal blastemas that later regress, and the complete blocking of regeneration that occurs after RNAi for genes such as *Smed-SmB* or *Smedinx-11*. These results suggest that different genes regulate different aspects of neoblast biology.

Other genes have been found to play a role in neoblast differentiation rather than in self-renewal or maintenance. This has been suggested for *Smedwi-2*, a homologue of the Piwi class of Piwi/Argonaute proteins (Reddien et al., 2005), *DjRbAp48*, a homologue of the retinoblastoma-associated protein 48 found in several chromatin-remodeling complexes (Bonuccelli et al., 2010), and *Smed-CHD4*, a homologue to the chromatin-remodelling proteins Chd4/Mi-2 (Scimone et al., 2010). In other systems, both RbAp48 and Chd4 are members of the nucleosome remodeling and histone deacetylase complex, which appears to play a pivotal role in regulating potency and differentiation (Crook, 2006; Ramírez and Hagman, 2009). It will therefore be important to characterize the function of chromatin-remodeling complexes in the regulation of neoblast proliferation and differentiation (Aboobaker, 2011).

It has recently been proposed that neoblasts close to the wound are more receptive to differentiation signals during regeneration (Wenemoser and Reddien, 2010). The nature of the signals that control neoblast differentiation within the blastema under these conditions remains largely unknown. However, it has been suggested that the epidermal growth factor receptor (Egfr) signaling pathway may play a role in regulating the differentiation of some cell types, mainly neuronal, during regeneration (Fraguas et al., 2011). Thus, silencing of *Smed-egfr-3*, a gene expressed in neoblasts and the CNS, mainly results in small blastemas in which certain non-neuronal cell types seem to differentiate normally, but in which the numbers of several neural cell types are significantly reduced (Fraguas et al., 2011). Moreover, unlike controls, in which neoblast proliferation is mainly restricted to the stump region outside the blastema, those reduced blastemas contain a significant number of mitotic cells (Fraguas et al., 2011). Similar results have been observed by inhibiting the extracellular signal-related kinase (ERK) pathway during planarian regeneration (Tasaki et al., 2011), and this led to the proposal that pERK is necessary to promote neoblast differentiation within the blastema (Tasaki et al., 2011). As ERK is known to act downstream of Egfr signaling in other systems, studies are now required to explore the relationship between these two pathways in regulating neoblast differentiation during planarian regeneration.

## Intercellular Signaling and Axial Pattern Formation in Planarians

Metazoans exhibit a highly diverse range of body plans, ranging from the apparently asymmetric organization of sponges to the bilaterally symmetric body plan of vertebrates and most invertebrates. The symmetry of bilateral animals is defined by three main perpendicular body axes, the anteroposterior (AP), the dorsoventral (DV) and the left-right, or mediolateral axis. The establishment of the body plan takes place during early embryogenesis. Consequently, most of our current understanding of the molecular mechanism that governs this event comes from embryonic models, mainly *Xenopus* and *Drosophila*. During regeneration, however, appropriate mechanisms are also required for the re-establishment of these axes. Yet, are those regulatory mechanisms the same that are active during development? What triggers their re-activation? When a planarian is cut into several pieces, how does each piece know whether to regenerate a head or a tail? In recent years, many of these questions have begun to be answered.

Classical studies using *Xenopus* embryos demonstrate that patterning requires the formation of an 'organizer' as a source of soluble factors capable of instructing differential fates in the surrounding territory according to their concentration (reviewed in De Robertis et al., 2000). These factors are known as 'morphogens'—although nowadays it is known that many of the secreted proteins from the organizers are not morphogens themselves but rather their modulators (activators or inhibitors). Bone morphogenetic proteins (BMPs) and Wnts form two of the main families of morphogens found in animals, and give their name to the so-called BMP and Wnt intercellular signaling pathways. Intercellular signaling cascades comprise not only the secreted elements (the ligands) but also the corresponding receptors and co-receptors, which, when activated by the ligands, trigger a cascade of intracellular protein phosphorylation that culminates with the activation of general transcription factors in the nucleus. Those transcriptional complexes will then activate and repress specific target genes responsible for conferring identity on the recipient cell.

Studies in several embryonic models demonstrate that the BMP and Wnt signaling pathways play an evolutionarily conserved role during the specification of the DV and AP axis, respectively (reviewed in De Robertis and Kuroda, 2004; Petersen and Reddien, 2009a; Niehrs, 2010). But, are they also responsible for axial re-specification during planarian regeneration? The answer is clearly yes. In recent years, several studies have been published reporting the role of the main elements of both pathways during planarian

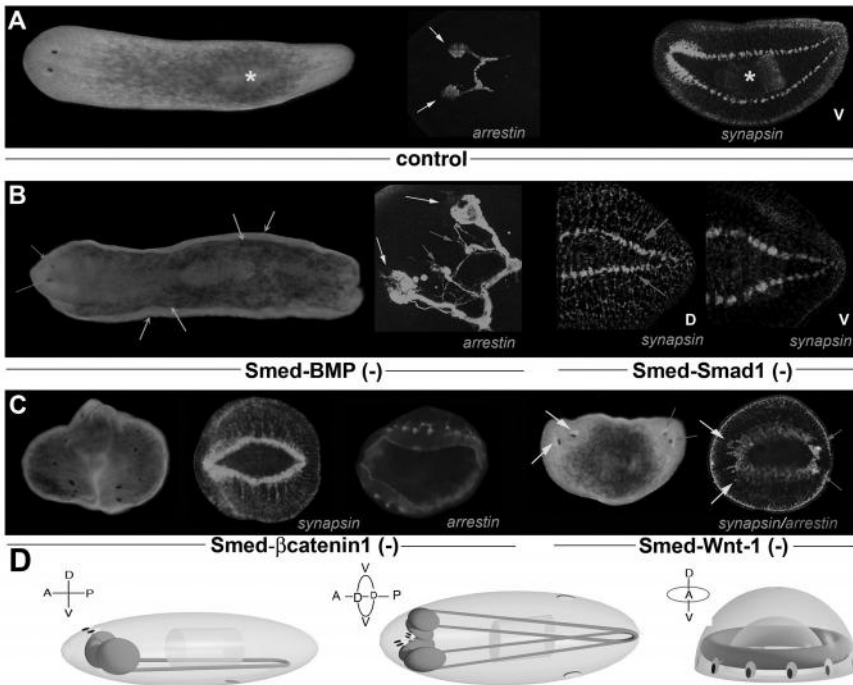
regeneration, clearly demonstrating the essential role of the Bmp and Wnt pathways in establishment of the DV and AP body axis, respectively, during planarian regeneration and homeostasis (reviewed in Adell et al., 2010; Molina et al., 2011a; Reddien, 2011; Almuedo-Castillo et al., 2012).

## **The BMP Pathway is Essential for DV Axis Re-establishment**

In animals, the DV axis is mainly determined by the action of the BMP pathway. Whereas in vertebrates *bmp* activation defines the ventral side of the animal, in invertebrates it specifies the dorsal side. These opposite functions, together with the fact that many conserved genes of the *bmp* pathway show opposite expression patterns along the DV axis, have led to the hypothesis that the DV axis has gone through an inversion between vertebrates and invertebrates during evolution (Arendt and Nubler-Jung, 1994).

The binding of secreted BMPs to a complex of type 1 and 2 transmembrane serine threonine kinase receptors triggers the phosphorylation and activation of a receptor-associated SMAD, which subsequently complexes with SMAD4 and translocates to the nucleus to regulate gene transcription. Several elements of the BMP pathway have been identified and functionally characterized in planarians (Orii and Watanabe, 2007; Molina et al., 2007, 2011b; Reddien et al., 2007, Gaviño and Reddien, 2011). Functional analyses have shown that BMP and SMADs (SMAD1 and SMAD4) are required for dorsal specification, as ventralized animals are produced after their silencing. This ventralization is clearly observed by the disappearance of dorsal molecular markers together with the ectopic expression of ventral ones on the dorsal side, as well as by the duplication of the eyes, the mouth, and the body margin (Figure 5). More remarkably, silencing of the BMP pathway results in the duplication of a central nervous system in the ventralized dorsal side of the treated animals (Orii and Watanabe, 2007; Molina et al., 2007; Reddien et al., 2007) (Figure 5). On the other hand, silencing of planarian *noggin*s, well-known antagonists of the BMP signaling, yields complementary dorsalized phenotypes in which ectopic outgrowths expressing dorsal markers develop ventrally (Molina et al., 2011b).

During *Xenopus* development, axial specification requires secretion of BMP modulators from a second organizing centre in addition to a ventral source of BMPs (De Robertis, 2009). This second dorsal center secretes the agonist anti-dorsalizing morphogenetic protein (ADMP), a member of the BMP family, and antagonists such as CHORDIN and NOGGIN. Together, these proteins form a complex self-regulatory circuit that restricts BMP activation to the ventral region of the embryo in order to specify those ventral fates. Even though this circuit has been shown to function in *Xenopus*, evidence of conservation of the pathways in invertebrates has only recently



**Figure 5. Phenotypes generated after silencing BMP and Wnt signalling pathway elements.** (A) Control planarians. (B) *Smed-bmp* and *Smed-Smad1*-silenced planarians show a ventralized phenotype: ectopic differentiation of eyes deep in the mesenchyme and ectopic differentiation of nerve cords in the dorsal region. (C) *Smed-βcatenin1* and *Smed-WntP-1*-silenced planarians show an anteriorized phenotype. Multiple eyes appear around a large circular cephalic ganglion in *Smed-βcatenin1*-silenced planarians, and a posterior head appears in *Smed-WntP-1*-RNAi animals. (D) Schematic drawing showing abnormal axial patterning after silencing of BMP or Wnt signaling. The antibody used for immunostaining is indicated in each image (anti-synapsin, which labels the CNS, and anti-arrestin, which labels the visual axons). Yellow asterisks indicate the pharynx and yellow arrows indicate the eyes. Red arrows indicate ectopic eyes. Orange arrows indicate duplication of the planarian margins after BMP silencing. D, dorsal view, V, ventral view. Anterior to the left. Modified from Cebrià et al., 2010.

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been obtained. Two independent studies have found that planarians have a conserved *admp* gene that is expressed on the ventral side opposite to the dorsal expression domain of *bmp*. More importantly, planarian BMP and ADMP appear to regulate each other in order to re-establish the DV axis during regeneration, similarly to the BMP/ADMP circuit described in *Xenopus* (Gaviño and Reddien, 2011; Molina et al., 2011b). In addition to providing evidence of a functional BMP/ADMP circuit during regeneration, planarian studies have led to the identification of a novel conserved regulatory element of the BMP pathway, the *noggin-like* genes, which differ

from canonical *noggin*s in a short amino-acid insertion within the *noggin* domain (Molina et al., 2009). Planarians have two canonical *noggin* and up to eight *noggin-like* genes (Molina et al., 2009). Surprisingly one of them, *nlg-8*, is expressed on the dorsal side of the animal, like *bmp*, and, when silenced, generates a BMP-like phenotype in which ectopic ventral markers appear dorsally and a duplication of the CNS is observed (Molina et al., 2011b). More remarkably, *noggin-like* genes are evolutionarily conserved, including in *Xenopus*, and their overexpression in frog embryos results in ventralized animals similar to those obtained following overexpression of *bmp* (Molina et al., 2011b). These data suggest that, in contrast to the canonical inhibitory function of *noggin*s, *noggin-like* genes might play a role promoting *bmp* activity during the establishment of the DV axis. Further studies both in planarians and *Xenopus* should help to elucidate the role of *noggin-like* genes within an already quite complex pathway.

### **The Wnt Pathway is Essential for AP Axis Re-establishment**

During planarian regeneration, anterior-facing wounds always regenerate new anterior regions, whereas posterior facing wounds always regenerate posterior regions. A question that has been puzzling the field for more than one hundred years is how planarians re-establish a proper AP axis during regeneration. The receptors of the Wnt pathway are the Frizzled proteins, together with LRP co-receptors, which after binding Wnt ligands lead to the disruption of the  $\beta$ -catenin degradation complex, composed of axin, Gsk3, Cki and Apc. As a consequence,  $\beta$ -catenin, which is the key intracellular element of the pathway, accumulates in the cytoplasm, enters the nucleus, and activates TCF transcription factors, which are able to regulate the expression of multiple genes (Gordon and Nusse, 2006).

The Wnt/ $\beta$ -catenin or canonical Wnt pathway (to distinguish from the  $\beta$ -catenin-independent or non-canonical pathway) has a strikingly conserved role in establishing the AP axis (Kiecker and Niehrs, 2001; Holland, 2002). Several elements of the Wnt/ $\beta$ -catenin pathway have been characterized in planarians and analysis of their function has revealed a pivotal role in the specification of the AP axis during regeneration (Gurley et al., 2008, 2010; Iglesias et al., 2008, 2011; Petersen and Reddien, 2008, 2009b; Adell et al., 2009). The most extreme phenotype is obtained after silencing the planarian homolog of  $\beta$ -catenin (*Smed- $\beta$ catenin1*). *Smed- $\beta$ catenin1*-silenced planarians appear as ‘radial-like hypercephalized’ animals, which have large circular cephalic ganglia that induce the differentiation of several ectopic eyes all around the planarian body (Iglesias et al., 2008) (Figure 5). In-depth molecular analysis reveals that those animals are fully anteriorized, since central and posterior identities are completely lost. Moreover, although the AP axis is completely abolished, the DV axis remains normal. Thus, disruption of the

Wnt/ $\beta$ -catenin pathway exclusively affects the establishment of AP polarity during planarian regeneration. The role of the Wnt/ $\beta$ -catenin pathway in AP axis patterning in planarians has also been demonstrated by silencing other elements of the pathway. For example, 'two-headed' animals are obtained following silencing of the planarian homolog of Dishevelled, an intracellular protein required for Wnt signal transduction, the homolog of *evi/wntless*, which is required for Wnt secretion, or Wnt-1 (Figure 5) (Gurley et al., 2008; Adell et al., 2009). In contrast, when *Apc-1* or *Axins*, components of the  $\beta$ -catenin degradation complex, are silenced, the opposite phenotype is obtained. Under these conditions, a tail differentiates instead of a head in regenerating planarians (Gurley et al., 2008; Iglesias et al., 2011). Taken together, these data demonstrate a conserved role for the Wnt/ $\beta$ -catenin pathway in AP axis establishment in planarians.

One thing that remains unclear is how the action of Wnt ligands is spatially regulated along the AP axis. It has been proposed that there is a gradient of  $\beta$ -catenin activity along the length of the planarian body, with its maximum concentration in the posterior region (Adell et al., 2010). According to this hypothesis, inhibition of *Smed- $\beta$ -catenin1* activity would lead all planarian cells to adopt an anterior fate. Is this gradient of  $\beta$ -catenin activity directly regulated by a gradient of a Wnt ligand? The morphogens of the pathway, namely the Wnt family, have been the subject of recent studies. Interestingly, of the nine Wnts found in the *S. mediterranea* genome, four are specifically expressed in the posterior part of planarians: *Smed-wnt-1*, *Smed-wnt11-1* and *Smed-wnt11-2* are located in only a few cells at the most posterior end of the animal, whereas *Smed-wnt11-5* is expressed in the posterior half (Petersen and Reddien, 2008; Adell et al., 2009). Accordingly, posterior defects are observed when these genes are silenced, although only Wnt1 inhibition is able to anteriorize the animals, resulting in 'two-headed' phenotype (Figure 5). Given that the rest of the planarian Wnts also show a very specific regional expression pattern, it has been suggested that multiple Wnts cooperate to pattern the different regions of the planarian body, as previously proposed for cnidarians (Guder et al., 2006).

Other mechanisms, such as intercellular communication system through gap junctions or the hedgehog (Hh) pathway are also known to be involved in establishing and maintaining AP polarity in planarians (Nogi and Levin, 2005; Oviedo et al., 2010; Rink et al., 2009; Yazawa et al., 2009). Thus, when the function of innexins, the gap junctional components of invertebrates (Nogi and Levin, 2005), is chemically blocked, 'two-headed' planarians are generated (Nogi and Levin, 2005; Oviedo et al., 2010). On the other hand, silencing of the planarian homolog of *Patched*, an Hh receptor with inhibitory activities, generates 'two-tailed' planarians, whereas inhibition of the Hh, *Gli-1* or *Smo* homologs induces anteriorization. In the last case, however, rather than a 'two-headed' phenotype, a 'no-tail' phenotype is



usually generated in which the posterior markers do not really disappear (Rink et al., 2009). Interestingly, simultaneous silencing of *patched* and  *$\beta$ catenin1* produces anteriorized animals, demonstrating that Hh acts upstream of  $\beta$ -catenin (Rink et al., 2009). In fact, the current functional data, and the strong phenotypes generated when modulating canonical Wnt signaling, suggest that the integration of information essential for AP patterning acts at the level of  *$\beta$ catenin1* activity.

In summary, current data indicate that, as described in different embryonic models, the Bmp and Wnt pathways also specify the DV and AP body axes, respectively, during planarian regeneration. Furthermore, they are required for maintenance of the body plan during normal planarian homeostasis. This means that the same mechanisms governing regeneration also govern normal homeostasis in adult planarians. Of course, this is not very surprising considering that planarians undergo continuous growth, degrowth and remodeling. It is nevertheless an important observation because it agrees with several studies using other animal models in which it became evident that developmental genes and functional networks are also active in adults. However, an extremely important question has not yet been addressed in depth in this very suitable model. Intercellular communication pathways are universal mechanisms not just to direct cell fate but also to control cell proliferation, tissue growth and regulation of stem cell maintenance and differentiation. In fact, patterning of an organism depends upon the coordination of cell growth, differentiation and morphogenesis. How do the Bmp and Wnt signals direct planarian cell proliferation and differentiation and, at the same time, control the differentiation and maintenance of the neoblast population? From the current data it seems that Wnts are not expressed in neoblasts, although irradiation experiments demonstrate that some of them require neoblast integrity for their expression during regeneration, suggesting that they would be expressed in stem-cell descendants (Gurley et al., 2010). Future studies should help to determine whether neoblasts express Wnt receptors, as one would expect, and how those signals regulate cell proliferation versus cell arrest, how they direct differential fates, and how growth and patterning are integrated.

## **ORGAN FORMATION DURING PLANARIAN REGENERATION**

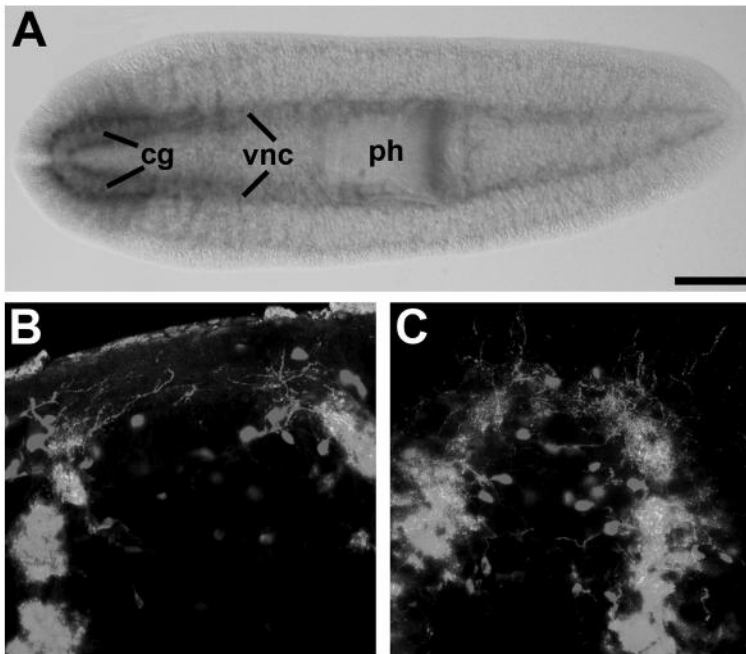
Organogenesis is the process of differentiation and patterning of each individual organ. Clearly, the general mechanisms controlling proliferation, differentiation, migration and patterning must be coordinated to generate such complex and perfect systems. As happens during embryogenesis, during regeneration the re-formation of every organ is preceded by the

differentiation of specific primordial structures derived from undifferentiated cells or stem cells. Very recent studies on the regeneration of specific organs during planarian regeneration have approached the question of whether the new cells that make up the primordial structures within the blastemas arise from a unique population of totipotent neoblast or, alternatively, there is a heterogeneous population of lineage-restricted neoblasts.

## The Structure and Regeneration of the Planarian Central Nervous System

Freshwater planarians are among the very few animals capable of regenerating their central nervous system (CNS). Despite their simple body plan, planarians possess a complex nervous system, both at the cellular and molecular levels (reviewed in Cebrià, 2007). Comparative studies have shown how planarian neural genes display a higher similarity to their homologues in vertebrates than to other model invertebrate species such as *D. melanogaster* and *C. elegans* (Mineta et al., 2003). In fact, genes expressed in the CNS that have been lost in *D. melanogaster* or *C. elegans* have been found to be conserved in planarians, which suggests that planarians are a better model for those comparative genomic studies designed to gain insight into the common evolutionary origin between the vertebrate and invertebrate CNS (Mineta et al., 2003). In addition, planarians provide us with an experimental framework in which to analyze the functions of all these conserved neural genes during the process of regeneration compared to the functions of their invertebrate homologues during embryonic development of the CNS.

The CNS of freshwater planarians consists of a pair of cephalic ganglia (forming a bi-lobed brain) connected usually by a transverse commissure and a pair of VNCs that extend along the length of the animal (Figure 6). The bi-lobed brain has a different shape in different species and this is usually related to the shape of the head (Cebrià, 2007). The VNCs are connected by transverse commissures that extend from regularly spaced knot-like ganglia to generate a ladder-like pattern. Since the VNCs also extend below the cephalic ganglia, they can be considered as distinct, though closely associated, structures from both a morphological and a molecular perspective (Agata et al., 1998; Cebrià et al., 2002). Recently, distinct neuronal subpopulations have been identified using commercial and specific antibodies in *S. mediterranea* and *Dugesia japonica*, the most commonly studied planarian species. Thus, currently available markers allow us to label dopaminergic, serotonergic, GABAergic, octopaminergic and cholinergic neurons, as well as neurons positive for anti-FMRFamide, -GYRFamide, -allatostatin and -neuropeptide F antibodies (Cebrià, 2008;



**Figure 6. Structure and regeneration of the planarian CNS.** (A) Whole-mount *in situ* hybridization for *Smed-pc2*, a pro-hormone convertase homologue, expressed in the cephalic ganglia (cg), ventral nerve cords (vnc) and the neural ring in the distal part of the pharynx (ph). (B-C) Regeneration of the planarian CNS detected by double immunostaining with anti-synapsin (in green, panneuronal) and anti-5HT (in red, serotonergic neurons) after 3 days (B) and 7 days (C) of regeneration. In (A), anterior is to the left; in (B-C), anterior is to the top. Scale bar in (A), 500  $\mu$ m.

*Color image of this figure appears in the color plate section at the end of the book.*

Nishimura et al., 2007a, b, 2008a, b, 2010; Fraguas et al., 2012). In addition, a genome-wide analysis to identify neuropeptide prohormone genes in *S. mediterranea* has confirmed the high level of complexity of its nervous system, which contains a large number of distinct neuronal cell populations (Collins et al., 2010). This array of markers can be used to generate a fine physical map of the planarian nervous system and also to analyze how all these neuronal cell types regenerate (Nishimura et al., 2007a, 2008a, b, 2010; Fraguas et al., 2012) (Figure 6).

After decapitation, planarians are able to regrow *de novo*, a fully differentiated and functional CNS in about 7–10 days. Within the regenerative blastema, a new brain develops and the truncated nerve cords grow to reestablish contacts with the cephalic ganglia. In the last 10 years, analyses of the expression patterns of a large collection of neural-specific genes together with RNAi-based functional analyses have led to the

proposal of a working model for planarian CNS regeneration (Cebrià et al., 2002; Cebrià, 2007; Agata and Umesono, 2008). Thus, the regeneration of this nervous system can be divided into three main stages: I.—brain primordia formation and patterning, II.—re-establishment of neural connectivity, and III.—functional recovery. Between one and two days after amputation, two bilateral clusters of cells corresponding to the primordia of the new cephalic ganglia are observed within the regenerative blastema (Cebrià et al., 2002; Kobayashi et al., 2007). The cell clusters then grow and differentiate into the new brain. As expected, the appearance of the brain primordia depends on the presence of neoblasts. As described in previous sections, irradiation kills the neoblasts and therefore irradiated planarians are incapable of producing a blastema and regenerating. Consequently, irradiated planarians do not produce brain primordia following decapitation. But what are the signals that commit the undifferentiated neoblasts to a neural fate? Despite several attempts to elucidate the underlying mechanisms, it remains unclear (Umesono and Agata, 2009). The planarian homologues of the *musashi* gene family, for instance, are expressed in neural stem and progenitor cells in other animals (Okano et al., 2005), and are expressed in post-mitotic differentiated neurons in planarians (Higuchi et al., 2008). Contrary to the expectations, however, silencing of planarian *musashi* genes does not affect brain regeneration (Higuchi et al., 2008). Taken together with their expression patterns, this suggests that *musashi* genes are not required for the commitment of the neoblasts to a neural lineage. Additional markers for neural stem and progenitor cells are therefore required, and the genes required for neural differentiation need to be found in order to understand the mechanisms through which the neoblasts generate the high diversity of neuronal populations observed in the planarian CNS.

At this early stage of CNS regeneration, the brain primordia appears to be already subdivided into the distinct molecular domains that can be distinguished in the mature differentiated brain. Thus, both the mediolateral and anteroposterior domains defined by planarian *Otx* genes (Umesono et al., 1999) and WntA and frizzled homologues (Kobayashi et al., 2007) are defined between one and two days of regeneration (Higuchi et al., 2008).

The second main stage in CNS regeneration (2–4 days of regeneration) involves growth of the brain primordia into mature cephalic ganglia and extension of the pre-existing amputated VNCs into the regenerative blastema. During this stage, the VNCs and the brain re-establish their proper connectivity so that the *de novo* differentiated brain takes control of the whole body. Although the interplay between the process of differentiation of the brain and the growth of the VNCs within the blastema remains poorly understood, at the very early stages of regeneration it appears that the brain primordia differentiate within the blastema before any outgrowth from the amputated VNCs are observed, at least according to currently

available markers (Cebrià et al., 2002). Since the two bilateral clusters of cells defining the brain primordia appear in front of the amputated VNCs, it is possible that signals coming from those cords determine the position at which the neoblasts start to differentiate into brain cells. Some studies have also suggested that the anterior commissure that connects the two cephalic ganglia develops by extending along neural processes that emerge from the VNCs and serve as a scaffold (Reuter et al., 1996). However, other recent studies have shown that at day 2–3 of regeneration some transverse commissures that connect the left and right brain primordia originate from those primordia; at the same time, though, other markers reveal the presence of fine transverse processes extending from the two VNCs (Fraguas et al., 2012). In summary, until we have specific markers to unambiguously distinguish between those processes emerging from either the brain primordia or the VNCs, we will not be able to have a clear understanding of the initial steps that allow re-establishment of the neural connections between the brain and VNCs.

What is known, however, is the function of several genes that play important roles in the wiring of the regenerated nervous system. Several studies have shown that conserved axon guidance cues such as *netrin* and *slit* play important roles during the regeneration of the planarian neural architecture. Netrins were originally identified as attractive cues for axonal growth in *Drosophila*, *C. elegans* and vertebrates, where they are important for guiding commissural and visual axons, among others (Ishii et al., 1992; Mitchell et al., 1996; Serafini et al., 1994, 1996). In planarians, silencing of the homologues of either a *netrin* or a *netrin receptor* results in disruption of the neural architecture both during regeneration and in intact animals (Cebrià and Newmark, 2005). As a result of silencing these genes, the VNCs do not grow as two parallel cords of compacted axonal processes but instead regenerate as a completely disorganized meshwork of processes. Moreover, the newly regenerated cephalic ganglia appear shorter, wider and connected by a thicker anterior commissure. In fact, it appears as if the central neuropil is more loosely packed compared to controls after the silencing of *netrin* or *netrin receptor* (Cebrià and Newmark, 2005). Finally, these two genes are also required for correct targeting of the visual axons. Planarian photoreceptor cells display a stereotypical pattern of axonal projections, with some axons projecting ipsilaterally towards the brain visual center, whereas others cross the midline to form an optic chiasm and project contralaterally (Okamoto et al., 2005) (Figure 5A). After silencing either *netrin* or *netrin receptor*, the visual axons fail to project ipsilaterally towards the brain visual center (Cebrià and Newmark, 2005).

*Slit* genes define another important family of conserved repulsive guidance cues. Both in *Drosophila* and vertebrates, *slit* mutants have a phenotype involving collapse of commissural and longitudinal axons at

the midline (Rothberg et al., 1990; Kidd et al., 1999; Long et al., 2004). In planarians, *slit* shows a conserved expression pattern along the midline (Cebrià et al., 2007). Remarkably, silencing of *slit* during planarian regeneration results in the collapse of the regenerating nervous system and photoreceptors at the midline. Moreover, ectopic neural tissues and photoreceptors differentiate along the midline of intact non-regenerating animals (Cebrià et al., 2007).

In addition to these secreted axon guidance cues, planarian cell adhesion molecules such as DjCAM and DjDSCAM appear to be required for normal morphology of the regenerated brain. When these genes are silenced, the central neuropil and the lateral brain branches appear rather defasciculated compared to controls (Fusaoka et al., 2006). Also, inhibition of a clathrin heavy chain (Chc) homologue in planarians disrupts the morphology of the regenerated CNS (Inoue et al., 2007). Analysis of cultured neural cells derived from Chc-RNAi animals reveals that they extend fewer and shorter neurites compared to controls (Inoue et al., 2007). Thus, *chc* may be important *in vivo* for axonal growth during neural differentiation. Finally, recent evidence suggests that planarian *wnt5* and *dishevelled* homologues have a role in the re-establishment of connections between the VNCs and the cephalic ganglia (Almuedo-Castillo et al., 2011). During regeneration, the new cephalic ganglia differentiate in a laterally displaced position with respect to the VNCs, while the VNCs regenerate as a disorganized axonal meshwork compared to the compacted axon bundles found in the two parallel cords in controls. This function of *wnt5* in neural network formation appears to be independent of  $\beta$ -catenin (Almuedo-Castillo et al., 2011).

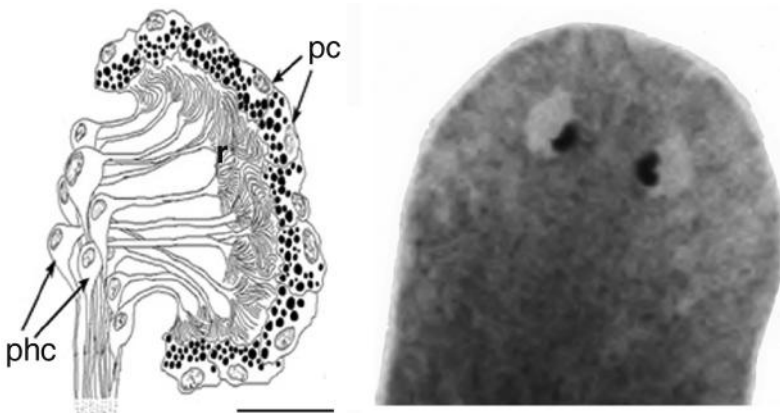
In summary, planarians are unique in their capacity to regenerate *de novo* a complex CNS through the differentiation of adult totipotent stem cells. Given the high level of conservation between planarian and vertebrate neural genes, these amazingly plastic animals might provide relevant insights for use in regenerative medicine to treat, for instance, neurodegenerative diseases or spinal cord injuries.

## Regeneration of the Eyes

In planarians, light is perceived by specific photoreceptor cells that form various eye types, mostly pigment-cup ocelli, located dorsally to the cephalic ganglia (Hyman, 1951). Most planarian species possess two eyespots (Saló and Batistoni, 2008). The presence of only two cell types (photoreceptors and pigment cells) defines the planarian eye as one of the most simple rhabdomeric eye types (Callaerts et al., 1999; Gehring and Ikeo, 1999). Photoreceptor cells are bipolar neurons with a rhabdomeric structure in which the microvillar dendrites containing the opsin photopigment are

enclosed in a monolayered cup of pigment cells (Figure 7). Pigment cells also act as phagocytes to remove photoreceptor membranes during the daily turnover of the visual cells (Tamamaki, 1990). The photoreceptor cell bodies are located just outside the pigmented eyecup and their axons project to the cephalic ganglia. The eye connection with the brain through a partial chiasma allows photosensory inputs to be integrated and processed, suggesting that complex neuronal circuits regulate planarian behavior (Figure 5).

Planarian eye regeneration is a unique model for the study of eye morphogenesis as it allows the molecular fate of the cells that make up the eyes to be traced. Despite the substantial morphological differences between invertebrate and vertebrate eyes, there is clear evidence that early morphogenesis involves a common gene network known to as retinal determination gene network (Treisman, 1999). This network includes genes belonging to the *Pax*, *Six*, *Eyes absent* (*Eya*) and *Dachshund* (*Dach*) families. Most of these genes have been identified and characterized in different planarian species (Saló and Batistoni, 2008). Thus, *Six-1* and *Eya* are required for eye regeneration in planarians, as “non-eye” phenotypes are obtained when the genes are silenced (Pineda et al., 2000; Mannini et al., 2004). Unexpectedly, silencing of *Pax6*, considered a master regulator gene for eye development in other systems, does not produce an eye phenotype (Pineda et al., 2002). However, transgenic planarians in which an artificial *Pax6*-dependent *opsin* promoter drives EGFP expression in photoreceptor cells confirmed the presence of *Pax6* or *Pax6*-related activity in the planarian photoreceptors (González-Estévez et al., 2003).



**Figure 7. Planarian eyes.** Planarian eye spots are located dorsally in the head. They are composed of two cell types. The photoreceptor cells (phc) have rhabdomeric termini (r), where the photopigment opsin accumulates, and axonal projections targeting the brain visual center. The pigment cells (pc) produce an inverted cup where the rhabdomeric structures accumulate.

Although classical transplantation experiments suggested that the planarian brain plays a key role in eye regeneration (Lender, 1950), we still do not know the origin of eye cells. Since planarians have totipotent stem cells, the source of new eye cells must be neoblasts. Several studies have therefore attempted to determine that site at which the eye precursor cells are determined and to trace their lineages. Evidence suggests that a complex combination of signaling molecules and transcription factors defines the eye cell lineage during eye regeneration and development. Interestingly, a gene related to the *nanos* gene family (*Smednos*), which has a conserved function in germline stem cell maintenance, is transiently expressed during the early stage of eye formation in *S. mediterranea*, both during development and regeneration. At later stages, when the eyes are completely differentiated, *Smednos* expression declines to undetectable levels, which suggest that it may play a role in the process of proliferation and maintenance of eye precursors to prevent their early differentiation to eye cells during regeneration (Handberg-Thorsager and Saló, 2007). Recently, two conserved transcription factors, the distal-less homeobox containing gene *Dlx* and the zinc-finger gene *Sp6-9*, have provided some clues about eye pigment cell determination and lineage. These genes are expressed from the early stages of eye precursor cell commitment until their final differentiation as pigmented cells (Lapan and Reddien, 2011). Silencing of *Dlx* and *Sp6-9* leads to selective loss of pigmented cells and mis-targeting of axonal projections from the photoreceptor cells. Co-expression analysis of these genes together with neoblast markers led to the suggestion that distinct segregated populations of precursors for both eye cell types exists long before they express terminal differentiation markers and aggregate in the newly regenerated eye-cup (Lapan and Reddien, 2011). This hypothesis does not fully agree with prior studies suggesting that photoreceptor and pigment cell types could be derived from a common ancestor that would co-express terminal differentiation markers of each cell type (Takeda et al., 2009). More detailed molecular analysis is required to solve this question.

Silencing of *Smed-egfr-1*, a gene also expressed in the eye pigment cells, impairs the differentiation of those pigment cells without affecting the number of photoreceptor cells (Fraguas et al., 2011). These results could indicate that both cell types derive from distinct precursor populations or that *Smed-egfr-1* is required for the terminal differentiation of pigment cells once they have segregated from a common precursor. Whether or not they share a common precursor, however, some studies suggest that both photoreceptor and pigment cells are necessary to form a normal eye-cup. Thus, after silencing of *Smed-egfr-1*, although the number of newly regenerated photoreceptor cells is not affected, they appear slightly disorganized compared to normal eyes (Fraguas et al., 2011).



The interdependence of the two eye cell types is also apparent when genes specific for photoreceptor cells are targeted. For instance, a planarian homolog of the homeobox-containing gene *orthodenticle* (*OtxA*), with an evolutionarily conserved role in patterning of the anterior head, is present in the photoreceptor cells of *D. japonica* and also appears restricted to a medial region of the cephalic ganglia, considered the putative visual center to which the photoreceptor axons project (Umesono et al., 1999). Interestingly, when this gene is silenced in *S. mediterranea*, photoreceptor neurons are completely lacking but an optic cup still differentiates, although it is smaller than in controls (Lapan and Reddien, 2011). A similar result was reported from studies addressing the role of the Bmp signaling pathway during eye regeneration. Recent expression analysis of several prohormone genes revealed the existence of three different subpopulations (anterior, posterior and ventral) of photoreceptor neurons in the planarian eyes (Collins et al., 2010). Interestingly, it has been shown that the BMP pathway plays an important role in regulating the number of anterior photoreceptor cells (González-Sastre et al., 2012). Thus, by silencing *Smed-smad6/7-2*, an inhibitory Smad (I-Smad) that represses Bmp activity, the anterior population of photoreceptors cells is lost. In contrast, inhibition of the Bmp pathway expands the population of anterior photoreceptors without affecting the number of the posterior cells (González-Sastre et al., 2012). Again, in those studies the disappearance or expansion of the anterior photoreceptor cell population correlates with a decrease or increase in the number of pigment cells.

Finally, a gene coding for opsin (a rhabdomeric opsin type)—a marker of terminal differentiation of photoreceptors—has been isolated in three planarian species (*Girardia tigrina*, *Gtops*; *S. mediterranea*, *Smedops*; and *D. japonica*, *Djops*). As expected, interference with *opsin* function by RNAi produces planarians with eyes that are non functional (Saló et al., 2002). Pigmented cells also express markers of functional differentiation, such as a specific tyrosinase (Lapan and Reddien, 2011) and tryptophan hydroxylase (Takeda et al., 2009; Fraguas et al., 2011). All these proteins represent useful molecular markers to follow differentiated eye cells.

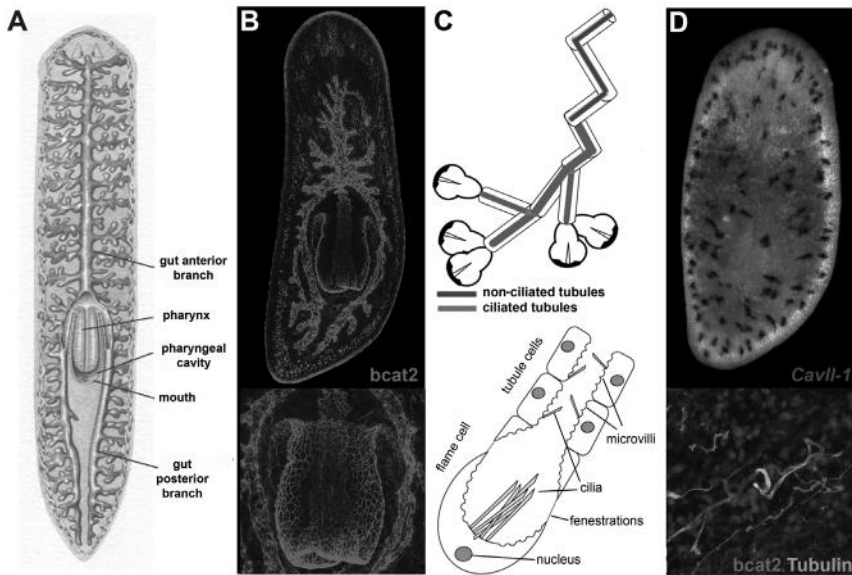
Taken together, current data support a scenario in which eye progenitor cells are independently determined in regions far from the eye differentiation field. The different lineages from the two eye cell types would finally differentiate and aggregate in a precise location and pattern, and interact with each other to produce a functional eye spot (Lapan and Reddien, 2011).

## Regeneration of the Digestive System

The digestive system is the part of the body dedicated to ingest and absorb nutrients. The uptake of the smallest particles into the body is essentially similar in all metazoans, and is performed by specialized cells, the phagocytes, through endocytosis. The uptake of larger particles requires prior chemical or mechanical breakdown. A second type of specialized cells, the secretory cells, are responsible for secreting the digestive enzymes that are required for chemical digestion. To facilitate the wide distribution of nutrients and increase the surface through which the nutrients are absorbed, a common feature of the digestive tract is its highly branched architecture.

The planarian digestive system includes a pharynx and a blind intestine formed by one main anterior and two main posterior branches, which ramify into secondary, tertiary and quaternary branches towards the lateral margins of the body (Figure 8). The three branches are connected to a contractile pharynx located in the middle of the planarian body. This pharynx is a highly innervated muscular organ that projects through a ventrally located mouth in order to take in food (Figure 8). The epithelial cells of the gut are organized into a single layer surrounded by a basal lamina and enteric muscles (Bueno et al., 1997; Kobayashi et al., 1998; Orie et al., 2002). Two main specialized intestinal cell types have been identified by histological analyses: phagocytes, which engulf food particles for their subsequent intracellular digestion, and secretory goblet cells, which secrete digestive enzymes into the lumen (Bowen, 1980; Garcia-Corrales and Gamo, 1988).

In contrast to other tissues, the epithelial digestive track presents a remarkable turnover rate in all metazoans. In mammals, the monostratified epithelium lining the intestinal tube is extensively folded and organized into millions of invaginations known as crypts. In those crypts reside a small number of specific stem cells able to replace absorptive as well as secretory differentiated cells. This stem cell-based adult system is highly active; it is estimated that the intestinal epithelium is completely replaced every 5 to 10 days. Specific stem cells that sustain the daily renewal of the midgut have been identified not only in vertebrates but also in invertebrates such as *Drosophila* (reviewed in Casali and Batlle, 2009). As one would expect, in planarians the digestive system is completely re-formed after any amputation. Furthermore, it was the analysis of this organ that clearly showed the extensive remodeling response of the pre-existing tissues according to new body proportions (Morgan, 1902; Gurley et al., 2010). The digestive tissues also show a continuous remodeling according to food availability and animal size in intact animals and during regeneration.



**Figure 8. Planarian digestive and excretory system.** (A) The digestive system consists of a blind diverticulated gut with one anterior and two posterior branches, a single pharynx in the center and a ventrally located mouth. (B) Immunostaining with anti-βcatenin2, which labels the adherens junctions of epithelial cells. The epithelium of the digestive branches and the different epithelial layers of the pharynx can be observed. (C) The excretory system is composed of long tubular structures, which are highly ciliated in their proximal parts. These connect to a specialized terminal cell, the flame cell, located inside the parenchyma. Adapted from Scimone et al., 2011 and Rink et al., 2011. (D) *In situ* hybridization for carbonic anhydrase (CAVII), which is expressed in the distal, non-ciliated part of the excretory tubules, and immunostaining with anti βcatenin2 and anti α-tubulin, which label the cell junctions of the tubular structures and the cilia, respectively (the nuclei are stained with DAPI, in blue).

*Color image of this figure appears in the color plate section at the end of the book.*

An important question is whether the totipotent neoblasts are the source of the new digestive epithelium or, as in other animals, there is a population of stem cells specifically fated to replace intestinal tissues. All the previous data in planarians indirectly suggested that neoblasts were the only source of new cells, as proliferating somatic cells have not been observed within any differentiated tissues. In the digestive system, this question was specifically addressed in a very recent study in which the intestinal branches were imaged in living planarians fed with fluorescently conjugated dextrans (Forsthoefel et al., 2011). In that study, the authors confirmed that no intestinal cells ever divide, supporting the notion that neoblasts are the sole source of new intestinal cells. The authors reported that during regeneration, as well as during homeostasis, mitotic cells are always found in the mesenchyme, often closely associated with the enteric

muscles, but never on the luminal side of the muscle boundary. Those data also show that, during regeneration, neoblasts are the source of intestinal cells not only within new intestinal branches that form in the blastema, but also in the pre-existing intestine that remodels far away from the wound. Irradiation experiments further demonstrate that neoblasts are absolutely required for intestinal reorganization after any amputation (Gurley et al., 2010; Forsthoefel et al., 2011). It remains unclear, however, what the signals are that launch and organize such a response of the neoblasts so far from the wound. Interestingly, it is known that extensive cell death occurs in tissue fragments that must remodel to re-establish the new proportions (Reddien and Sánchez Alvarado, 2004; Pellettieri et al., 2010). An interesting possibility, therefore, is that this cell loss triggers the proliferation of the neoblasts.

The source of adult intestinal cells in planarians is different from that found in other models, in which intestinal cells arise from specialized stem cells located in the same epithelium. In planarians, the neoblasts, located in the mesenchyme, must commit to intestinal cell fates, then cross the muscular layer and the basement membrane, and finally integrate into the intestinal epithelium and differentiate (Forsthoefel et al., 2011). This raises the question of which signals trigger this behavior in the neoblasts and whether the intestine releases cues to promote neoblast lineage restriction and migration. Furthermore, it remains to be seen whether the signals involved in stem cell maintenance and differentiation in other models are conserved in planarians' gut replacement. The Wnt pathway, for instance, is one of the best known signaling pathways required for enteric stem cell maintenance. Mice lacking Wnt signaling in the intestinal epithelium lose the crypt progenitor compartment, whereas constitutive Wnt activation results in a massive expansion of crypt stem cell number and tumorigenesis (reviewed in Van der Flier and Clevers, 2008). In fact, a mutation in the *Apc* gene, a key activator of the Wnt intracellular response, is one of the first events leading to human colorectal cancer (Markowitz and Bertagnolli, 2009). The picture that emerges from recent analysis of *Drosophila* intestinal stem cells also reveals an essential role for Wnt signaling (Casali and Batlle, 2009). It will therefore be interesting to determine whether Wnt signaling plays a role in controlling the fate of digestive cell precursors or pluripotent neoblasts.

## Regeneration of the Excretory System

The excretory organs have two main functions: 1) regulation of the salt concentration of the organism in relation with the medium (osmoregulation) and 2) elimination of toxic metabolites from the body. One of the basic principles occurring in excretory systems is the 'ultrafiltration' of the

interstitial fluid within the extracellular matrix, which takes place in the so called 'nephridia'. The planarian excretory system is composed of 'protonephridia' located all along the body margins (Figure 8). The protonephridia consist of branching tubules ending in one terminal cell or flame cell, which is completely surrounded by extracellular matrix and is the site of ultrafiltration (Ruppert and Smith, 1988; Bartolomaeus and Ax, 1992). It is generally assumed that the concerted beating of the flame cell cilia generates a negative pressure that forces fluid from the extracellular space to the lumen of the flame cell across the extracellular matrix of the cell fenestrations (Wilson and Webster, 1974).

Few studies have been published about this system in planarians or in other invertebrates. For instance, although it is commonly accepted that dorsally located nephridiopores would connect protonephridia to the outside (Wilhelmi, 1906; Hyman, 1951), their existence has never been proved. Fortunately, two studies recently reported detailed data on the structure and regeneration of the planarian excretory system (Rink et al., 2011; Scimone et al., 2011). Using ultrastructural analysis as well as new molecular markers that specifically label different parts of the protonephridia, both laboratories showed that the planarian excretory system consists of a very organized and complex tubular structure of specialized epithelial cells, in which one or two terminal ciliated cells (flame cells) are always found underneath the epidermis, immediately below the muscular layer that surrounds the mesenchyme. Terminal cells continue with the proximal tubules, which are also ciliated, and these branches grow into the mesenchyme. The more distal tubules are found deeper in the mesenchyme and are non-ciliated (Rink et al., 2011; Scimone et al., 2011) (Figure 8).

As one of the functions of the excretory system is osmoregulation, inhibition of genes essential for its integrity leads to bloating and lysis of the animals. Functional analysis by RNAi revealed several genes associated with this kind of disorder, and further analysis revealed a role in regeneration and maintenance of the planarian excretory system. For instance, the *Egfr* pathway seems to be required for epithelial integrity in the protonephridia (Rink et al., 2011). Silencing of a planarian *Egfr5*, which is expressed in proximal segments and in flame cells, causes morphological defects in proximal tubules and a reduced number of flame cells. Also, conserved transcription factors such as the Pou homeobox gene *Pou2/3* and *Six1/2-2*, specifically label proximal tubules and distal tubules respectively, and are found to be essential for their regeneration (Scimone et al., 2011).

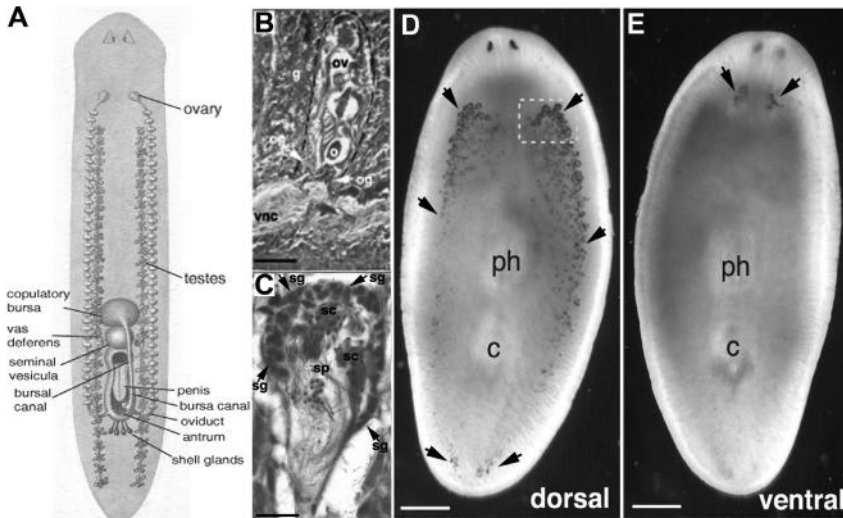
Analysis of specific markers for the protonephridia by *in situ* hybridization at specific time points after amputation demonstrates that the regeneration of the new excretory system follows a very specific sequential program. Furthermore, the new protonephridia do not appear to arise from the elongation of the old one but from the differentiation of

precursor structures inside the blastema that later connect to the preexisting system (Rink et al., 2011). A recent model proposed by Scimone et al. (2011), suggested that a transient population of nephridia precursor cells co-expressing *Six1/2*, *Pou2/3*, *Sall*, *Eya1* and *Osr1* are derived from neoblasts (*Smedwi-1* and *H2B* positive). These precursors appear to constitute a heterogeneous population of cells that co-express markers of pluripotency and markers of nephridial fate. Later, these nephridia precursor cells would differentiate as tubule or tubule-associated cells by expressing different combinations of genes. Taken together, these data support a very similar scenario to the one already described above for eye organogenesis. After amputation, neoblasts would migrate to the wound and begin to differentiate into the missing cell types. At this time point, we would be able to observe a heterogeneous population of neoblasts that co-express markers of pluripotency and markers of specific differentiated cell types. Later on, cells will reach the differentiated state and form the final organized structures, losing the potential to divide and differentiate into different cell types (Scimone et al., 2011).

## Regeneration of the Reproductive Organs

Freshwater planarians can reproduce sexually or asexually, by transverse fission. During asexual reproduction, planarians split their tail, and both resulting fragments regenerate the missing tissue producing two planarians, one much smaller than the other. In the sexual mode, they behave as hermaphrodites and cross-fertilize before laying egg capsules that contain several embryos (cocoons) (Figure 1). The reproductive organs of sexual planarians consist of numerous dorsolaterally distributed testes and two ovaries located posterior to the cephalic ganglia (Newmark et al., 2008; Chong et al., 2011) (Figure 9). An individual lobe of the testes has an outer layer of spermatogonia that undergo three rounds of division with incomplete cytokinesis, generating eight primary spermatocytes that progress through meiosis to generate 32 spermatids (Franquinet et al., 1973; Chong et al., 2011). Testis are interconnected by the sperm ducts, which run along the nerve cords and conduct mature sperm to the seminal vesicles and the copulatory apparatus, which is located in the region posterior to the pharynx. From the ovaries, two oviducts also run along the nerve cords to transport fertilized eggs to the copulatory apparatus and the genital pore (Newmark et al., 2008) (Figure 9).

Two distinct mechanisms have been described to specify germ cell fate in animals: determinate specification, in which cytoplasmic factors localized in specific regions of the egg play the main role (observed in *Drosophila*, *Caenorhabditis* or *Xenopus*), and epigenetic specification, which requires inductive interactions between cells (observed in many basal metazoans



**Figure 9. Planarian reproductive system.** (A) The planarian sexual strains have a complex reproductive system with two ventral ovaries, two dorso-lateral lines of testis and vitelline glands. The copulatory apparatus contains a copulatory bursa (where the sperm exchanged during copulation is stored), a penis, a seminal vesicular (where the mature sperm is stored), and shell glands that connect to the atrium to produce the polyembryonic eggs, or cocoons. (B) Sagittal paraffin section of an ovary (ov) located in between the digestive system (g) and the ventral nerve cord (vnc). Oögonia (og) are at the base and in the periphery of the ovary. The mature ova (o) are in the lumen of the ovary. (C) Sagittal paraffin section of a testis. The spermatogonia (sg) are located in the periphery of the testis. Maturation of the spermatogonia takes place approaching the lumen of the testis and gives rise to the spermatocytes (sc) and the matured sperm (sp) in the lumen. In the sperm duct leading away from the testis, sperm can be observed. (D) and (E) Whole mount *in situ* hybridization with *Smed-nanos*, which is expressed dorsally in the testes (D) and ventrally in the ovaries (E). Scale bars, 1 mm. Adapted from Handberg-Thorsager and Saló (2007).

and also in mammals) (Newmark et al., 2008). In sexual planarians, gonads and the copulatory apparatus develop in their definitive regions when the animal reaches a certain size (Newmark et al., 2008). Thus, the second mechanism, requiring the induction of adult cells, appears to govern germ cell specification. Furthermore, during adulthood, planarians are able to regenerate their germ cells *de novo*. For instance, the entire reproductive system of planarians is resorbed when the animals shrink due to starvation (Schultz, 1904; Berninger, 1911), or after severe amputation (Wang et al., 2007). Only when they reach the appropriate size and completely rebuild their somatic structures do the reproductive organs redifferentiate. Interestingly, it seems that the testis are only reformed after regeneration of the cephalic ganglia is complete (Ghirardelli, 1965), suggesting that inductive influences from the brain could be essential for sexual maturation.

Recent findings certainly demonstrate that specific prohormones expressed in the nervous system of sexual planarians, and not in asexual animals, are required for the development and maintenance of mature reproductive organs (Collins et al., 2010).

Are the neoblasts also the source of newly regenerated germ cells in planarians? All the reported data would argue that the answer is 'yes'. The main experiments to address this question are based on the use of irradiation to kill the neoblasts. Irradiation leads to regression of the testes and ovaries, which cannot be regenerated when lethal doses are used. More than 20 years ago, it was demonstrated that the transplantation of cell fractions, enriched in neoblasts from sexual strain of *S. mediterranea*, into lethally irradiated asexual planarians was able to restore their capacity to regenerate both somatic and germ cell lineages (Baguña et al., 1989). Although it cannot be ruled out that the enriched cell fraction contains precursor germ cells, it is currently assumed that planarian germ cells can be regenerated *de novo* from somatic stem cells. These features provide us with a unique model in which to study the nature of the inductive signals responsible for the specification of germ cell fate. In an effort to identify those signals, the main intercellular signaling pathways have been studied in asexual races during regeneration, although fewer data are available on their function in the development of the germline in sexual animals. Most of the molecular studies performed on sexual planarians are focused on identifying specific genes expressed in germ cells and required for their specification. The most specific and interesting gene identified so far is *Smed-nanos*, a zinc-finger protein with a widely conserved role in the maintenance of germ cell identity. In sexual planarians, *nanos* RNA is detected from late embryonic stages, when the primordial reproductive organs are formed, up to the sexually mature stage. During regeneration, their expression in the pre-existing germ cells is maintained, and during the first week it is restored in the newly formed tissue (Sato et al., 2006; Handberg-Thorsager and Saló, 2007; Wang et al., 2007). Functional studies demonstrate that *nanos*-RNAi animals regenerate their somatic tissues normally but are not able to regenerate the reproductive system (Wang et al., 2007). Similarly, in *nanos*-RNAi-silenced sexually mature planarians, the testes and ovaries degenerate and *nanos*-RNAi newly hatched planarians fail to develop testes or ovaries (Wang et al., 2007). These results clearly indicate that *nanos* is required for the postembryonic development, regeneration, and maintenance of planarian germ cells, as described in all other metazoans.

It is interesting to note that *nanos*-RNAi is also detected in asexual strains as positive dorsolaterally located cell clusters, in the same position as the testes primordia in hermaphrodites (Sato et al., 2006; Handberg-Thorsager and Saló, 2007; Wang et al., 2007). RNAi experiments in asexual animals demonstrate that *nanos* is required for the regeneration and



maintenance of these presumptive germ cells, suggesting that asexual planarians are able to specify the germ line.

Germ cells are differentiated cells that retain the capacity to generate all cell types of an organism. Not surprisingly, they share several morphologic and genetic features with stem cells. This raises the question of the extent to which the neoblasts are similar to planarian germ cells. When analyzed by electron microscopy, they are morphologically indistinguishable. Their cytoplasm contains electron-dense ribonucleoprotein particles known as chromatoid bodies, which were first described as a germ cell feature (Auladell et al., 1993; Kotaja and Sassone-Corsi, 2007). Furthermore, several genes that have conserved expression in germ cells are also expressed in neoblasts. These include homologs of *piwi*, *vasa*, *pumilio*, and *bruno* (reviewed in Newmark et al., 2008). In agreement with the common features shared by germ and stem cells, even *nanos*, the most specific germ-line gene found in planarians to date, is also transiently expressed in precursors of the differentiated cells which form the eyes, both during development and during regeneration (Handberg-Thorsager and Saló, 2007). Although several *piwi* paralogs are found in planarians and most of them are expressed in different subpopulations of neoblasts, a *piwi* gene was recently reported to be specifically required for the development of germ cells but not somatic cells or even somatic sexual organs (Nakagawa et al., 2012).

## CONCLUSION

The capacity of planarians to completely regenerate any missing part of their body is dependent upon a unique type of stem cells, the neoblast, which can give rise to every adult cell type. According to the most recent insights from molecular and cellular studies, neoblasts form a heterogeneous population that probably includes a subpopulation of true totipotent stem cells and a subset of partially determined neoblasts that retain their proliferative capacity. Whether the regeneration of missing tissues involves only the totipotent population or a combination of totipotent and partially determined neoblasts is an important question that will require further studies. A question that requires further attention is the origin of these adult stem cells. Analysis of the Bmp and Wnt pathways reveals that they are permanently activated in planarians, which therefore resemble eternal embryos. It remains to be determined, however, whether the high activity of the intercellular signals leads to the occurrence of totipotent stem cells or whether totipotent stem cells bring about permanent activation of the signaling pathways. What is clear is that planarians are an emerging model system with great potential for the understanding of regeneration, continuous morphogenesis and the biology of stem cells.

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## ABBREVIATIONS

|      |                                       |
|------|---------------------------------------|
| VNC  | ventral nerve cords                   |
| CNS  | central nervous system                |
| BMP  | bone morphogenetics protein           |
| ADMP | antidorsalizing morphogenetic protein |

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# Zebrafish in Stem Cell Research

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## SUMMARY

The zebrafish is becoming an increasingly significant model for stem cell research. With unique physical attributes that allow for optically transparent, high-throughput screening and experimentation, the zebrafish affords unique *in vivo* analysis of stem cell behaviors, regeneration, chemical screening, and transplantation. Additionally, the growing number of techniques available for genetic manipulation of the fully sequenced zebrafish genome adds to the whole organism and *in vitro* utility of this vertebrate model. Large-scale forward genetic screens conducted in zebrafish continue to illuminate an abundance of novel mutations in genes involved in normal development and disease. This chapter will outline the natural characteristics that make zebrafish a valuable model organism, the tools and techniques available for a broad-range of stem cell-based investigations, and future directions for this promising field.

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*List of abbreviations after the text.*

## INTRODUCTION

The use of zebrafish in biological research began at the University of Oregon in the late 1960s and has since developed into a model system used worldwide by investigators studying vertebrate development, organogenesis, and human disease. *Danio rerio*, a common tropical freshwater fish, has proven advantageous for scientific research due to a number of physical attributes and genetic tools that make it amenable to high-throughput screens and *in vivo* analyses.

In a practical sense, the small size, ease of care, and relatively low cost of zebrafish make them accessible for labs with any degree of spatial or financial constraint. From a research perspective, the value of zebrafish comes from a host of morphological characteristics that allow for unique experimentation unparalleled by other vertebrate models. Due to the optical transparency of the zebrafish embryo and an adult mutant that lacks pigmentation, visualizing biological events *in vivo* throughout development and adulthood affords unprecedented insight into a broad range of stem cell behaviors, including migration, fate mapping, lineage tracing and niche interactions. Such transparency also makes the fish a unique model for stem cell and cancer stem cell transplantation assays. The rapid development and extraordinary fecundity of the zebrafish provide a platform for quick, high-throughput genetic and chemical screens that aim to identify genes and small molecules modulators involved in stem cell biology. Lastly, the amazing regenerative capacity of the zebrafish can be used to help understand the cellular and genetic mechanisms underlying a host of poorly understood stem cell behaviors.

In addition to a number of advantageous morphological traits, ongoing work with the zebrafish genome makes the organism increasingly useful for diverse genetic manipulations. Forward genetic screens performed with zebrafish have already identified thousands of mutations that affect hundreds of genes involved in development and disease, while reverse genetic technologies have afforded investigators the opportunity to study previously known genes of interest in a unique vertebrate model amenable to *in vivo* analysis and experimentation.

Given the high degree of homology between the zebrafish and human genomes, the ease with which zebrafish can be used for genetic and chemical manipulation and screening, and the growing collection of tools available for a broad spectrum of investigations, zebrafish-based research holds significant promise for stem cell laboratories of all sizes.

## ZEBRAFISH AS A MODEL ORGANISM

The zebrafish was originally selected as a model for genetic study on the basis of three distinctive traits: first, the relative ease of maintaining their prodigious breeding in the laboratory setting year-round; second, an external fertilization system; and lastly, an embryonic transparency that offers investigators the opportunity to view the developing fish *in vivo* and screen for specific phenotypes associated with normal develop, as well as disease (Grunwald and Eisen, 2002). Since its initial selection, the many unique morphological traits belonging to the zebrafish have secured its status as an emerging major model organism and continue to advance a variety of scientific investigations. This section will explore the biological characteristics that make the zebrafish a valuable model for stem cell research.

Like most organisms, zebrafish respond to the quality of their environment. The density of their housing, quality of food and water, regularity of feeding, and frequency of matings all contribute to fluctuations in breeding capacity. Thus, a single female zebrafish of fertile age can lay anywhere between 50–1000 eggs every week (Westerfield, 2000). The potential for extraordinary fecundity is especially useful when a large number of samples are required for experimentation. With the recent development of a device called the iSpawn, the number of zebrafish embryos available for research can be increased to upwards of 10,000 (Adatto et al., 2011). By mimicking the physical conditions in which zebrafish naturally mate, the iSpawn utilizes relatively few adult fish yet yields thousands of developmentally synchronized embryos over a ten-minute breeding window. The ability to generate thousands of equally staged embryos is integral for the success of large-scale zebrafish screens and is of great utility for similarly precise, high-throughput research.

While their breeding potential affords large-scale experimentation, it's the unique transparency of the developing zebrafish embryo that enables the complete visualization of such experiments with unprecedented ease. The transparency of the zebrafish embryo, coupled with cellular and subcellular live imaging, has greatly advanced the *in vivo* analysis of a number of physiological processes. Notable among such research is that of the hematopoietic system, where hematopoietic stem cells (HSCs) have been traced during emergence, differentiation, migration, and transplantation in the living organism (Bertrand et al., 2010; Renaud et al., 2011a; Traver et al., 2004; Yaniv et al., 2006a; Yaniv et al., 2006b).

Recently, a double mutant extended the transparency of the zebrafish into adulthood (White et al., 2008). *Casper*, a translucent, sexually viable, stable transgenic line has been particularly important in studies of HSC transplantation, where viewing the different fluorescent proteins that mark host versus donor tissues and cells *in vivo* significantly aids in measuring engraftment. Being able to observe the live interactions of a stem cell with its niche over time at any stage of development or adulthood is a major advantage for those studying stem cell behavior.

In addition to producing a large quantity of translucent embryos, the rapid pace of zebrafish development allows for quick generation of stable lines and study of the emerging cells, tissues, and systems within them. The approximate developmental stage of an embryo can easily be determined under a dissecting stereomicroscope using transmitted illumination and relatively low magnification (5–20X). With full visualization, one can observe a newly fertilized egg transition from the zygote period (0 hours), where the first cleavage will occur about 40 minutes post-fertilization, into the following sequential staging periods: blastula (2¼–5½ hours), gastrula (5¼–10 hours), segmentation (10–24 hours), pharyngula (24–48 hours), hatching (48–72 hours), and early larval (3 days). The fish reach sexual maturity by 10–12 weeks and adulthood in approximately 3 months.

Particularly important for stem cell research is the early emergence of the zebrafish organ progenitors, which can be detected within 24 hours post-fertilization (hpf) and primitive organs, such as the brain, eye, heart, pronephros, notochord, and somites, which can be observed by 36 hpf (Kimmel et al., 1995). The zebrafish heart begins beating after 22 hpf, with circulation of blood occurring around 24 hpf, and reaches its mature rhythm of 180 beats/minute by 36 hpf, a pace more similar to the human heart beat than that of the mouse (Stainier et al., 1993).

The zebrafish embryo is remarkably permeable, allowing for microinjection of transgenes as well as entrance of small molecules during chemical screens. Embryonic zebrafish are surrounded by three membrane-limited compartments: attached to the developing fish is a large yolk sac that is itself surrounded by the yolk syncytial layer; the differentiating blastoderm cells are surrounded by an enveloping layer similar to mammalian trophectoderm; and lastly, an outermost chorion that encapsulates the entire entity (Hagedorn et al., 1997).

Apart from the many morphological and biological traits that make zebrafish an advantageous model organism for stem cell research, they are also a practical model organism for labs of all sizes and budgets. The small size of a fully-grown zebrafish (3–4 cm) allows for easy housing in compact laboratory settings and their cost of maintenance is significantly lower than other vertebrate systems. Fantastic resources for the establishment and preservation of a zebrafish facility can be found in “The Zebrafish

Book: A Guide for the Laboratory use of Zebrafish (*Danio rerio*),” published online at [http://zfin.org/zf\\_info/zfbook/zfbk.html](http://zfin.org/zf_info/zfbook/zfbk.html) and in print through a number of accessible sources (Westerfield, 2000), in addition to “Zebrafish: A Practical Approach” (Nusslein-Volhard and Dahm, 2002) and “Modular, Easy-To-Assemble, Low-Cost Zebrafish Facility” (Kim et al., 2009).

Given the ease of viewing a single cell develop into an organism with rapidly forming tissues and organs physiologically similar to humans, in addition to being amenable to genetic manipulations and chemicals screens, the zebrafish is an advantageous model for studying stem cells under normal or genetically- or chemically-perturbed environments.

## **GENOME MODIFICATIONS**

This section focuses on methods for creating permanent modifications within the zebrafish genome that can be used to identify mutations in genes involved in development and disease, as well as to generate stable transgenic lines. Discussed in the first part are methods for “knocking out” genetic information from the native zebrafish genome, including: forward genetic screening, Targeted Induced Local Lesions in Genomes, zinc-finger nucleases, and transcription activator-like effector nucleases. The second part contains information for generating stable transgenic lines, which are necessary for addressing a multitude of stem cell investigations explained in the later sections of the chapter.

### **Knock Outs**

Information gathered from removing native DNA from the zebrafish genome have made the organism a valuable tool in the search for and exploration of regulatory pathways involved in a number of stem cell systems and processes. From screening for random mutations to targeting known genes, techniques that knock out genetic information are valuable for a wide range of experimental questions.

### **Forward Genetic Screening**

Information gathered from forward genetic screens has proven immensely significant for establishing the zebrafish as a major model organism. This subsection will explore a few of the screening techniques that brought zebrafish to the forefront of developmental biology and contributed to the understanding of key genetic pathways.

George Streisinger’s landmark paper was the first to establish the suitability of zebrafish for genetic analysis and screening (Streisinger

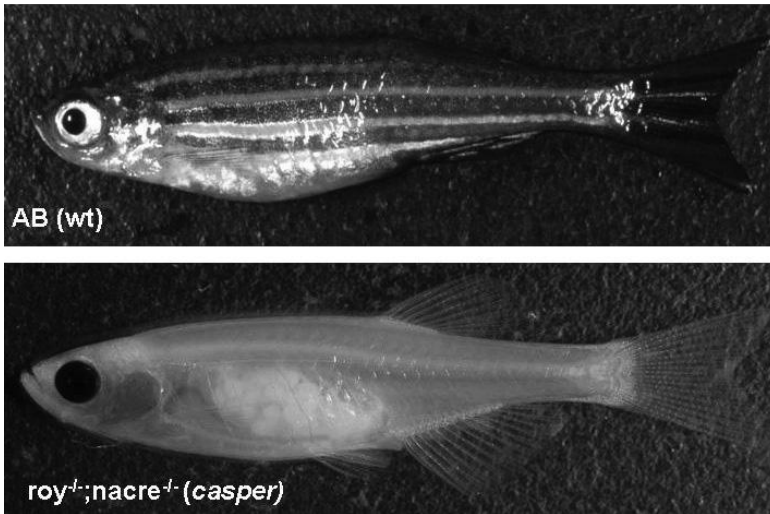
et al., 1981). Before this paper, genetic studies on vertebrates proved extraordinarily cumbersome, given the difficulty of detecting recessive mutations in diploid eukaryotes. Streisinger et al. worked around this issue by establishing methods for producing haploid and diploid gynogenotes in the zebrafish and creating clonal strains that were devoid of lethal mutations in their background. The scientists predicted their zebrafish clones and associated genetic methods would facilitate analysis of vertebrate genetic biology.

The first large-scale genetic screens that propelled zebrafish on to the stage of major model organisms were conducted in Boston, Massachusetts, and Tübingen, Germany, in the mid-1990s (Driever et al., 1996; Haffter et al., 1996). Known jointly as the “Big Screen,” these ventures were the first genetic screens to be performed in a vertebrate organism. Additionally, using zebrafish afforded unbiased observations of genes involved in development and disease in a model system more closely related to humans than had been previously studied in similar forward genetic screens performed on *Drosophila* (Barbazuk et al., 2000).

Between the two zebrafish screens, a total of 4000 embryonic lethal mutant phenotypes were discovered and published together in issue 123 of the journal *Development* after the genetic and preliminary phenotypic characterizations had been completed. This open source of information continues to provide scientists with a vast array of mutants that are relevant to many aspects of vertebrate embryonic development.

These initial screens were conducted using ethyl nitrosourea (ENU), a chemical that causes point mutations throughout the zebrafish genome, to induce mutations in the germ line of the ENU-treated males, which were then passed to heterozygous individuals in the  $F_1$  generation through pair wise matings of the mutagenized male with wild-type females (Mullins et al., 1994; Solnica-Krezel et al., 1994). The  $F_1$  and  $F_2$  generations were successively incrossed to obtain an  $F_3$  generation. In the presence of a recessive mutation, half of an  $F_2$  family should be heterozygous for that mutation. When two  $F_2$  fish are mated and both are heterozygous for the mutation, their progeny will display 25% wild-type, 50% heterozygous, and 25% homozygous genotypes. By mapping the mutations generated from this screening technique, called an  $F_2$  diploid screen, valuable information has been illuminated that helped identify interacting genetic pathways relating to formation of the zebrafish endoderm (Stainier, 2002), heart and blood (Thisse and Zon, 2002), and embryonic patterning (Hammerschmidt et al., 1996b; Mullins et al., 1996).

However useful  $F_2$  diploid screens have been identifying mutants and establishing the zebrafish field, their downfall is that they can prove disappointing when a mutation with an interesting phenotype is difficult to clone. A perfect case in point is the *cloche* mutant, who exhibits a loss



**Figure 1. wild-type and transparent mutant zebrafish.** A wild-type zebrafish (top) and a compound roynacre double homozygous mutant, casper (bottom), whose loss of melanocytes and iridophores results in a largely transparent fish uniquely amenable to *in vivo* experimental read outs (adapted from white et al., 2008).

*Color image of this figure appears in the color plate section at the end of the book.*

of endocardium in the heart and blocks blood cell differentiation at a very early stage yet whose locus in the genome remains elusive (Stainier et al., 1995).

To help facilitate rapid cloning of mutations, another type of large-scale diploid screen was conducted using insertional mutagenesis (Amsterdam et al., 1999). This approach created mutations by injecting retroviruses into the 1000–2000 cell stage embryo. The integration of exogenous DNA sequences into the zebrafish genome served as both the mutagen and tag, allowing for disruption of the genome and an easy avenue to identify where the disruption occurred. While expediting the cloning process, insertional mutagenesis screens are less efficient at mutagenizing the genome than chemicals and the inserted mutagens seldom, if ever, integrate entirely randomly into host DNA (Spradling et al., 1995).

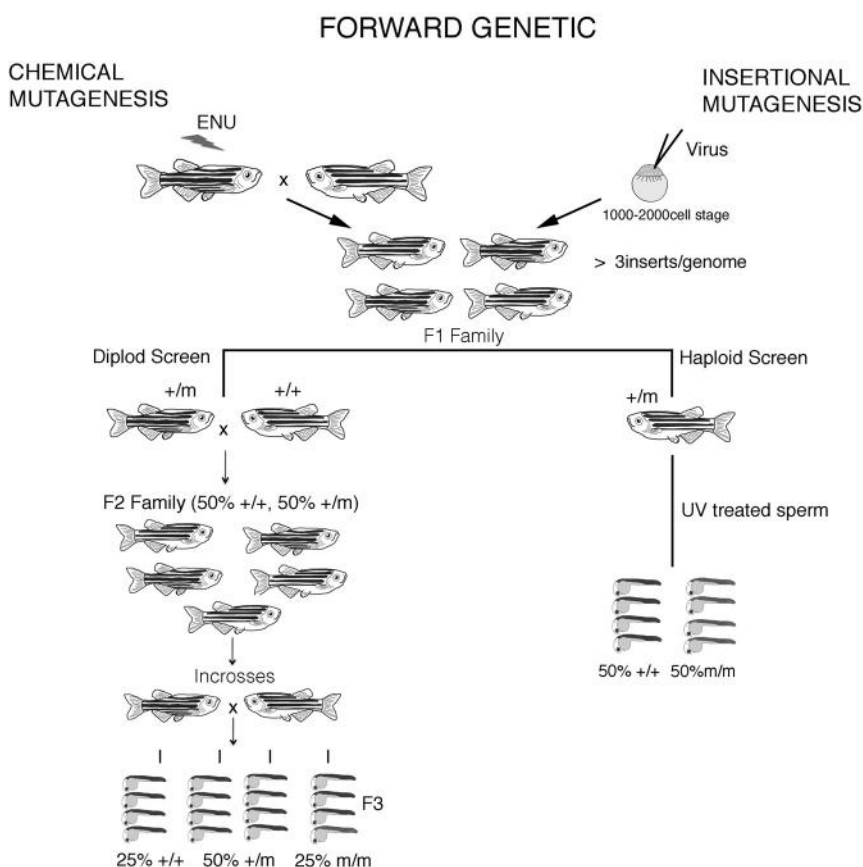
Other approaches that have proven useful for genetic screening include those that use gamma- and X-rays to mutagenize haploid zebrafish by generating large deletions and translocation events (Walker, 1999). In addition, gynogenetic inheritance screens, wherein the embryo develops under the guidance of the female genome only, can be conducted in either haploid (Cheng and Moore, 1997) or diploid (Beattie et al., 1999) organisms. This uniparental approach further simplifies the screening process by allowing a recessive mutation to be uncovered in one generation since



it will not be masked by the genetic contribution of the wild-type parent (Draper et al., 2004).

As an example of how a mutated fish with an interesting phenotype can be mapped to understand biological pathways during development, the mutant *kugelig* (*kgg*) may be used to highlight the applicability and potential of forward genetic screens in stem cell research.

Homozygous mutant *kgg* embryos develop abnormal antero-posterior (AP) patterning resulting in severe tail defects and acute anemia within 1 dpf (Davidson et al., 2003; Hammerschmidt et al., 1996a). Blood cell numbers recover by 5 dpf but the mutant embryos will die by 10 dpf. From mapping of this mutation, it was discovered that *kgg* mutants exhibit their embryonic



**Figure 2. forward genetic screening.** Diagram depicting chemical and insertional methods for genetic disruption, both resulting in a mutagenized f1 generation whose mutation can be further analyzed by diploid (left) or haploid (right) screening (courtesy of c. santoriello).

*Color image of this figure appears in the color plate section at the end of the book.*

lethal phenotype because of a mutation in the *cdx4* gene, belonging to the caudal family of homeobox genes implicated in antero-posterior patterning (Griffin et al., 1998). Hox genes are known to play a pivotal role in AP patterning but only overexpression of *hoxb7a* and *hoxa9a*, but not *hoxb8a*, can rescue the *kgg* mutant phenotype, suggesting that only certain hox genes are involved in hematopoietic cell fate specification (Davidson et al., 2003). The additional failure of *scl* overexpression, a transcription factor that is essential for the development of all hematopoietic lineages, to rescue the mutant *kgg* phenotype implies that *cdx4* and its associated hox genes are required for the posterior mesoderm to become “competent” for blood formation. Analysis of mutants discovered from forward genetic screens, like *kgg*, are valuable tools for discovering genes involved in vertebrate development and using such information to map genetic programs. In addition to *kgg*, a list of mutants that have been used to describe the various stages of hematopoietic maturation can be found in the review by (de Jong and Zon, 2005).

While forward genetic screens have identified mutants that are being used to unravel development processes, there are limitations to relying on this approach alone. Should a mutation not present a phenotype, for example, a potentially important gene can go unnoticed. To address this issue, it is necessary to employ methods of targeted analyses. In zebrafish, there exist a number of reverse genetics techniques that allow for such research. The remainder of this subsection focuses on techniques that allow for detection of recessive mutations in the genomic DNA of heterozygous or homozygous individuals, irrespective of any phenotype they may or may not present. With the sequencing of the zebrafish complete, analyses by tools listed in this section will become increasingly more valuable.

### **Targeted Induced Local Lesions in Genomes (TILLING)**

The process of Targeted Induced Local Lesions in Genomes (TILLING) has been applied to a range of species in order to discover rare mutations in non-mutant populations. The two main methods of detection for these polymorphisms are through Cel1 enzymatic activity and by resequencing. Cel1 is a plant-specific extracellular glycoprotein that cleaves heteroduplex DNA at all possible single nucleotide mismatches (Oleykowski et al., 1998). By using fluorescently labeled primers to detect Cel1 cleavage, it is possible to detect heterozygous single nucleotide polymorphisms (SNPs) and use these 1 kb fragments to screen for mutations in the genome (Colbert et al., 2001).

The second option for generating a screen of SNPs is through resequencing, wherein single mutagenized fish are resequenced at targeted gene loci (Wienholds and Plasterk, 2004). Both methods have been applied

to zebrafish to generate over a hundred loss-of-function mutations that are expected to provide insights in mechanisms of development and disease (Draper et al., 2004; Moens et al., 2008; Weienholds et al., 2003; Wienholds et al., 2003). In addition to identifying mutations in an arbitrary gene, TILLING also provides an allelic series of mutations. In a relatively unbiased way, many amino-acid substitutions, splicing mutations and stop mutations can be identified by TILLING (Stemple, 2004).

## **Zinc Finger Nucleases**

A that may facilitate more sophisticated manipulations of the zebrafish genome, uses zinc finger nuclease (Zfn) technology to knock out genes from the zebrafish genome. Zfns are chimeric fusions between a Cys<sub>2</sub>His<sub>2</sub> zinc finger protein (Zfp) and the cleavage domain of FokI endonucleases (Kim et al., 1996; Porteus and Carroll, 2005a, b). By engineering the Zfps to recognize a sequence of interest, the targeted cleavage will occur and result in a double-stranded break at the specified region (Hurt et al., 2003; Wright et al., 2006a; Wright et al., 2006b). As the cell attempts to repair the induced damage via non-homologous end joining (NHEJ), this error-prone repair pathway will introduce mutations in the region with the targeted gene, thereby knocking out its function (Beumer et al., 2006).

Once Zfns are designed and validated, their mRNA is injected into a single-cell stage embryo and the zebrafish is allowed to mature to adulthood, where it will be genotyped to verify the knock out event. The external fertilization of the zebrafish embryos makes them easily accessible for this type of injection and experimentation has shown that introducing these lesions early in development, such the single-cell stage, allows the mutation to be transmitted through the germline (Meng et al., 2008). The bottleneck that is currently inhibiting this technology's growth is the lack of validated Zfn libraries. Once a genome-wide collection of tested Zfns is established for the zebrafish community, it is imagined that such fingers could be virtually placed on the genome database and used to easily generate new knockout animals (Egger, 2008). The Zinc Finger Consortium is working to make this a reality by developing an open-access platform for engineering customized zinc finger arrays through modular design and selection ([www.zincfingers.org](http://www.zincfingers.org)).

## **TALENs**

The emergence of a recent technology may allow for germ line genetic disruption in the zebrafish without the laborious screening that Zfns require in order to identify a DNA binding domain with the desired specificity. This

technology was first introduced in two *Nature Biotechnology* articles that reported successful genomic modifications using transcription activator-like effectors (TALEs) in human cells (Miller et al., 2011a, b; Zhang et al., 2011a). Miller et al. discovered that TALE truncation variants from *Xanthomonas*, when linked to the catalytic domain of the FokI enzyme, could cleave DNA with an efficiency of 25% and be used to generate discrete edits or small deletions within endogenous genes (Miller et al., 2011a). Zhang et al. refined the technology by illuminating a method for the easier synthesis of new TALE variants that allow for full-length, customizable recognition of specific DNA-binding sites and demonstrating how these variants can be used to regulate endogenous gene transcription (Zhang et al., 2011b).

The use of TALENs (transcription activator-like effector nucleases) was soon extended to zebrafish for disruption of genes in somatic cells (Sander et al., 2011) and as well as in the germ line (Huang et al., 2011). Huang et al. further improved upon the technology by creating an alternative, simpler method for constructing TAL effector repeats called “unit assembly,” which involves the isocaudamer restriction enzymes, NheI and SpeI (Huang et al., 2011).

The demonstrated simplicity of TALEN design, which allows these nucleases to be constructed in high-throughput using an automated system the Joung lab has developed, in addition to its high rate of germ line transmission have made the system favorable over Zfns for the knock out of zebrafish genes. Additional work by the Joung lab has revealed the essentially limitless gene targeting potential of TALENs, further establishing its utility in the field of zebrafish gene modification (Reyon et al., 2012).

## Transgenesis

While knocking out a gene helps to identify its role in the development of the organism, inserting those that are previously known is equally educational. Indeed, genes that were discovered in other organisms are given new opportunities for study in the zebrafish, as the fish’s transparent, external development allows for an exceptional *in vivo* readout of the gene’s function. Additionally, creating transgenic fluorescent lines is necessary for a number of stem cell investigations, including the tracing of individual cells. Insertion of transgenes into the zebrafish genome is readily achieved by microinjection, wherein the gene of interest is introduced into the single-cell embryo with a micropipette (protocol found in (Xu, 1999)). There are three methods currently in use that promote the integration of exogenous genes into the zebrafish germ line. Presented in order of their efficiency in generating stable transgenic lines, these are: injection of “naked” DNA, injection using transposable elements, and site-directed transgenesis. To

find a list of the numerous ways transgenic zebrafish developed from all three methods are being used in science, refer to the searchable literature in the Zebrafish Model Organism Database, at [www.zfin.org](http://www.zfin.org).

## **DNA Injection**

The original method for generating transgenic zebrafish is the naked injection of genetic material into the single-cell stage embryo (Stuart et al., 1988). In this technique, plasmid DNA is linearized with a restriction enzyme, purified, then microinjected into the cytoplasm of the single-cell. While this method remains the easiest to implement, it is far from efficient with only a small percentage of injected embryos transmitting the transgene to the next generation. Additionally, these transgenes tend to form tandem arrays or concatamers at the integration site, which in some cases may lead to variegated expression or silencing in the following generation (Suster et al., 2009). Despite its low germline transmission frequency and transgene expression reliability, naked DNA injection has been successful in generating a number of influential transgenic lines; however, the addition of transposable elements when injecting plasmid DNA has significantly accelerated the efficacy of zebrafish transgenesis.

## ***Tol2*-mediated transgenesis**

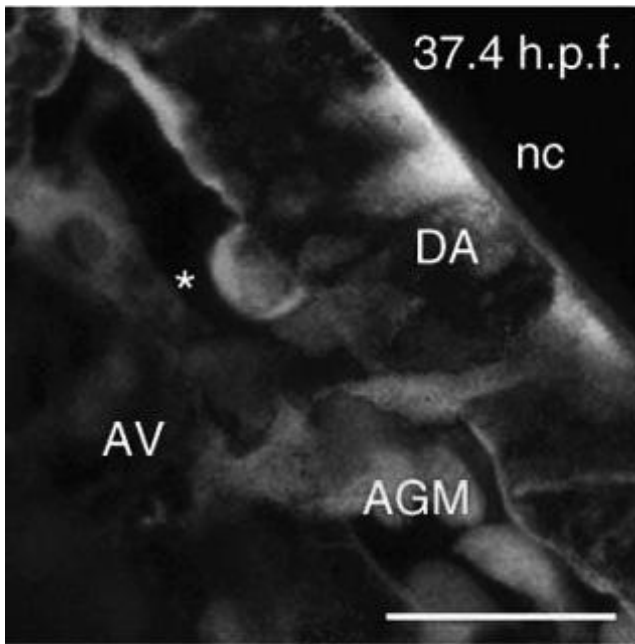
A host of transposable elements have been successfully co-injected with plasmid DNA into the zebrafish embryo in order to generate transgenic organisms but one stands out as the most reliable, reproducible, and efficient: *Tol2*. A transposable element isolated from the medaka fish, *Tol2* has the highest rate among transposable elements for genomic integration of exogenous DNA into the germ lineage (50–70% transmission rate from generation to generation) and is now a widely used genetic tool (Kawakami, 2007; Urasaki et al., 2006).

In this system, *Tol2*-donor plasmid DNA and the transposase mRNA are microinjected into the cytoplasm of fertilized, single-cell zebrafish embryos (Abe et al., 2011). Once inside the cell, the transposase protein is synthesized from the injected mRNA and will catalyze excision of the *Tol2* construct from the donor plasmid. The excised *Tol2* construct then randomly integrates into the genome during embryonic development. If the *Tol2* is inserted into germ cells, the transgene will be passed on to future generations.

The zebrafish *Tol2* system has undergone a series of improvements since its inception, including: the generation of vectors that facilitate rapid construction of promoter- or protein-GFP fusions through Gateway® technology (Villefranc et al., 2007); others that allow for the study of gene

expression and mutagenesis using *Tol2* gene and enhancer trap pairings (Kawakami et al., 2004); a targeted approach for specific tissues and cells that employs the *Tol2* system in conjunction with binary Gal4-UAS (Inbal et al., 2006); and its paired usage with Cre/*lox* technology, in addition to Cre<sup>ERT2</sup>, for tissue- and time-specific transgene induction (Mosimann and Zon, 2011). A useful resource for generating transgenic zebrafish with the use of transposable elements like *Tol2* can be found in Methods of Cell Biology (Clark et al., 2011).

Given the ability to easily create customized, reproducible, germline-transmitting transgenics, *Tol2*-mediated transgenesis has become a staple within the zebrafish community. Many transgenic animals generated from the *Tol2* system have been used in studies of stem cell behavior in the zebrafish. Notable amongst such investigations relate to hematopoietic stem cells (HSCs), whose visible emergence from the ventral wall of the dorsal aorta was an important discovery in the identification and isolation of the



**Figure 3. hematopoietic stem/progenitor cells in the vascular niche of the zebrafish embryo.** the caudal hematopoietic tissue (cht) is a vascular plexus in the tail of the embryo between the caudal artery and vein. it is the primary site of definitive hematopoiesis in the embryo, making it comparable to the mammalian fetal liver. shown is the cht of a 48 hour double transgenic embryo soon after it has been colonized by the first stem cells. hematopoietic stem/progenitor cells (green; *cd41:egfp*) reside in a niche of closely associated endothelial cells (red; *flk1:dsred*). note: anterior left and dorsal top (courtesy o.tamplin).

*Color image of this figure appears in the color plate section at the end of the book.*

stepwise intermediates that transition the aortic haemogenic endothelium into nascent HSCs (Bertrand et al., 2010). Without the transgenic fluorescent tags on these cells, their emergence and migration would not have been possible to witness *in vivo*.

## Site-directed Transgenesis

The majority of the aforementioned techniques for generation of transgenic zebrafish have relied on random genomic integration of the transgene. This lack of specificity can lead to transgene integration in sites that are prone to silencing or overexpression as well as leaving the animal vulnerable to multiple integrations that will disrupt Mendelian inheritance in future generations. To address these issues, a number of site-directed transgenesis techniques have been developed with the hope of controlling gene expression in the zebrafish. These include the aforementioned Gal4/UAS system as well as three site-specific recombinases (SSRs): Cre/*loxP*, Flp/FRT, and phiC31.

The Gal4/UAS system has been combined with gene- and enhancer-trap pairings to generate transgenic fish expressing the Gal4 transcription activator in specific cells, tissues, and organs in order to activate transgene expression (Asakawa et al., 2008; Davison et al., 2007a, b; Scott et al., 2007). By injecting constructs containing a modified version of the Gal4 transcription activator gene, the gene of interest, and transposable mRNA into fertilized embryos, then crossing these fish upon adulthood to UAS: GFP reporter fish, Gal4 transgene insertion and expression are identified as GFP expression in the offspring (Abe et al., 2011). Methods for transient gene expression, generation of stable transgenic lines, isolation of gene- and enhancer- trap fish expressing fluorescent proteins, and targeted gene expression studies with the Gal4-UAS system can be found in *Transgenesis Techniques* (Suster et al., 2009).

Cre (cyclization recombination), a 38-kDa protein from bacteriophage P1, can recombine target DNA recognition sequences called *loxP* sites to generate site-specific transgenesis as well. The *loxP* site is a 34-bp DNA sequence that consists of two 13-bp inverted repeats, which act as binding sites for Cre, that flank an asymmetrical 8-bp core region. Depending on the orientation and location of the recombinase recognition sites, Cre can catalyze excision, integration, inversion, or translocation of DNA segments. This system was first applied to zebrafish in 2004 and is described in further detail in (Dong and Stuart, 2004).

To further diversify the tools available for zebrafish transgenesis, a second site-specific recombination system, Flp/FRT, has been successfully implemented in the zebrafish (Wong et al., 2011). Flp (flipase) is a 43-kDa protein from the 2- $\mu$ m circle of *Saccharomyces cerevisiae*. Like Cre, Flp can

recombine 34-bp DNA recognition sequences called FRT (Flp recognition target) sites, consisting of two imperfect 13-bp inverted repeats flanking an asymmetrical 8-bp core region. In the case of both SSR systems, zebrafish embryos that carry either a *loxP*- or FRT-flanked EGFP expression cassette are injected with a capped version of the Cre or Flp mRNA, respectively, and recombination events are allowed to occur. While Cre and Flp can catalyze intermolecular recombination, the two *loxP* or FRT acceptor sites can also immediately recombine and reverse the integration event. To address this problem, a third SSR system has been established in the zebrafish using phiC31 integrase (Lister, 2010).

Unlike Cre/*loxP* and Flp/FRT, this system has two distinct acceptor sites, *attL* and *attR*, that cannot be acted upon by the integrase alone, which greatly decreases the chance of a reverse integration event (Smith et al., 2010). Such technology has been applied to achieve transgene excision in somatic (Lu et al., 2011) and germ cells (Lister, 2010) in the zebrafish. A part from excision, the phiC31 system has recently been reported for phiC31 integrase-catalyzed integration of transgenes as well (Hu et al., 2011).

Generation of transgenic animals becomes especially important in the following sections, where techniques that allow for the visualization, transplantation, and chemical screening of stem cells and cancer stem cells relies heavily upon the use of transgenic animals.

## STUDYING STEM CELL BEHAVIOR IN ZEBRAFISH

The unparalleled optical transparency of the zebrafish, coupled with the ability to create fluorescently labeled cells and tissues, offer unparalleled opportunities for visualizing stem cell migration, fate tracing, and niche interactions within the organism during normal development, regeneration, and transplantation. This *in vivo* analyses of embryonic and adult stem cell behaviors were unprecedented in vertebrate model organisms and have already been established in the study HSCs (North et al., 2007), neural crest stem cells (Dorsky et al., 1998a, b; White and Zon, 2008), and neural stem cells (Chapouton et al., 2006a; Chapouton et al., 2006b; Stigloher et al., 2008). During development, these stem cells migrate to their respective niches and undergo a series of self-renewal and differentiation events that will lead to the formation of tissues and organs, most of which can be viewed live in the zebrafish.

A number of optical imaging techniques exist that can be used for studying stem cell behavior in the zebrafish. These include transmission electron (Rieger and Koster, 2007), digital scanned laser light sheet fluorescence (Keller and Stelzer, 2010), and two-photon excitation time-lapse microscopy (Carvalho and Heisenberg, 2009), in addition to laser-scanning



(Renaud et al., 2011b), spinning disk (Ma et al., 2011), and resonant-scanning confocal microscopy (Williamson and Hiesinger, 2010).

## Migration

A well-documented study of stem cell migration in the zebrafish is that of the hematopoietic system. Zebrafish embryonic hematopoiesis greatly resembles its mammalian counterpart, including the presence of primitive and definitive waves (de Jong and Zon, 2005). The primitive wave gives rise to lineage-committed erythroid and myeloid progenitors only, while the definitive wave produces functional HSCs and all mature blood lineages in adults (Li and Zon, 2011). In studying HSC emergence and migration, there exist a number of techniques that can be readily applied to other stem cell systems for similar purposes.

For example, the *de novo* visualization of definitive HSCs emerging from the ventral wall of the dorsal aorta (DA), migrating to the previously-uncharacterized posterior blood island (PBI), then reaching their final destination in the adult kidney can be observed and validated through the use of transgenic lines, laser-activated cell labeling, whole-mount fluorescent in situ hybridization, and confocal microscopy (Jin et al., 2007). These experimental procedures that led to this discovery can easily be applied to other stem cells for similar analyses and will be expanded upon below.

First, the ability to fluorescently label the tissues that surround HSCs in a stable transgenic, in this case vasculature, in a different color than the stem cells let the researchers visually distinguish the HSCs from their pathway of migration. This two-color fluorescence staining technique (as described in (Jin et al., 2006) allowed for the *in vivo* observation of stem cells during migration.

Next, by using caged fluorescein dextran (CF) injection (Kozlowski et al., 1997) to spatiotemporally label individual cells, the migration of definitive HSPCs from the DA to the PBI was illuminated within the animal and observed by confocal microscopy. Using CF allows the researcher control of which cells to label and when to activate the fluorescence.

To validate the HSCs' identities, a technique called whole-mount in situ hybridization (WISH) was used to detect sites of specific gene expression, in this case genes that are exclusively expressed by definitive HSPCs (Thisse and Thisse, 2008). Jin et al. used WISH to identity stem cells in their fixed microenvironments in order to confirm and quantify the stem cells (Jin et al., 2007).

A more comprehensive review of available tools and protocols used to study HSC behavior in the zebrafish can be found in (Goessling and North, 2011), while a recent outline of the methods required for tracing

stem cell migration in the zebrafish can be found in the review by (Li and Zon, 2011). Labeling techniques similar to those used by Lin et al. have allowed for observation of migratory events in neural stem cells (Grandel et al., 2006), retinal stem cells (Raymond et al., 2006), and cardiac progenitor cells (Smith et al., 2008). Many of the methods described here for use in stem cell migration will be revisited in the study of fate mapping, lineage tracing, niche interactions, and regeneration in the zebrafish.

## **Fate Mapping & Lineage Tracing**

There exist a number of different ways to identify groups of cells involved in the development of a tissue or organ, called fate mapping; likewise, these techniques may be used to follow the fate at a single cell or even subcellular resolution, called lineage tracing. Whichever the goal of the experiment, the tools available for such investigations include a host of photosensitive proteins, in addition to caged fluorescein dextran and the use of SNAP-tag technology.

The fluorescence spectrum of photosensitive proteins can be easily converted with UV excitation to allow a single cell or population thereof to be distinguished from its neighbors. Currently, Eos (Mathur et al., 2010), PAmCherry (Subach et al., 2009), Kaede (Ando et al., 2002), pKindling (Chudakov et al., 2003), and KikGR (Habuchi et al., 2008) have been used to irreversibly label cells with significant success and ease. A labeled cell can be traced throughout development and will pass its fluorescence on to its daughter cells, which can help determine the cell's role in the organism. A potential downside to many photosensitive proteins, however, lies in their inability to sustain fluorescence for long-term cell tracking (Stark and Kulesa, 2007).

A more robust option for cell tracing is with the use of caged fluorescein dextran (CF), a fluorescent dye that is bound to a quenching group that masks its fluorescence until the "uncaging" event occurs (Kozlowski and Weinberg, 2000). CF can then be released from the quenching group by uncaging a specific cell using UV light from a laser or mercury lamp, thereby allowing visualization by fluorescence or immunodetection (Clanton et al., 2011). Generating zebrafish that exhibit this staged tracing dye begins with injection of CF into the embryo by standard microinjection techniques previously described in this chapter. Uncaging the dye can be accomplished through three primary methods: an epifluorescence microscope equipped with a pinhole, a single photon UV laser, or, most recently, a titanium sapphire femtosecond laser. This last method is a notable because, unlike UV, the near-infrared laser pulses produced from this laser do not disrupt biological tissue, which allows for uncaging of cells within a single

focal plane (Kohli et al., 2011; Niemz, 2003). Lasers of this final class are customarily included on most two-photon confocal microscopes.

Lastly, a recent technique has emerged allowing for labeling of subcellular structures, such as the nucleus, cell membranes, and endosomal membranes in zebrafish (Campos et al., 2011). This method uses SNAP-tag protein, a 20-kDa mutant of the human *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase, which can specifically react with *O*<sup>6</sup>-benzylguanine derivatives carrying a variety of different synthetic fluorophores to activate fluorescence in subcellular structures (Keppler et al., 2003). Benefits of this monomeric tag include its attachment to a wide variety of proteins and ability to label in all cellular compartments of both live and fixed cells. Additionally, detection of the SNAP-tag fusion can be achieved at any desired wavelength due to a large variety of unique, stable fluorescent substrates.

## **Stem Cell—Niche Interactions**

Given the niche's role in the supervision, maintenance, and differentiation of stem and progenitor cells, understanding its architecture and behavior cannot be understated in stem cell research. The niche mediates the response of stem cells to the needs of organism and is thus empowered with the dual potential to protect the organism, by regulating stem cell number and fate, or cause harm by allowing aberrant over-proliferation of stem cells, which in turn may lead to disease (Fuchs et al., 2004). Since these microenvironments can exhibit remarkable variability in number and function over the course of an organisms' life, the study of how these sites appear, go dormant, and migrate within tissues can help scientists understand these regions of stem cell regulation (Scadden, 2006). In light of the dynamic nature of niche biology, illuminating the mechanisms that bestow these specialized microenvironments with their unique abilities is central to understanding basic cell biology as well as targeting of potential therapies for a host of stem cell-associated diseases.

The zebrafish has become an increasingly important model for such investigations due to the many morphological traits and genetic tools that allow for visualization, screening, and genetic- and chemical- manipulations of niche biology. In contrast to mammals, stem cell niches in regions like the brain are abundant in zebrafish yet their origin, architecture, and function remain remarkably similar, making zebrafish an advantageous model for the study of stem cell-niche interactions (Kaslin et al., 2008). Additionally, whereas mammals cease the development of new neurons shortly after birth, neurogenesis continues over the entire span of the zebrafish lifecycle, making the fish's cerebellum a prolific site for stem cell activity and a rich source for stem cell research (Grandel et al., 2006).

Using transgenic lines, *in vivo* imaging, and marker analysis, among other techniques previously described in this section, novel stem cell niches have been elucidated in the zebrafish and used to understand common characteristics of vertebrate niche biology. Zebrafish have been notable in advancing the study of retinal (Raymond et al., 2006), hematopoietic (North et al., 2007), neural (Kaslin et al., 2009), and spermatogonial (Nobrega et al., 2010) stem cell niches. Their extension to other stem cell niches is promising.

## Regeneration

An exciting field of zebrafish research that has made landmark advances in understanding a particular aspect of stem cell biology is that of regeneration. Thus far, the zebrafish fin (Johnson and Weston, 1995), heart (Poss et al., 2002), retina (Bernhardt et al., 1996), optic nerve (Becker and Becker, 2002), spinal cord (Becker et al., 1997), liver (Sadler et al., 2007), sensory hair cells (Lopez-Schier and Hudspeth, 2006), and brain (Kroehne et al., 2011) have all been studied for their regenerative capacities.

Upon injury, the zebrafish begins regrowth of various tissues by epimorphic regeneration, a term used to describe a regenerative process involving the formation of a mass of undifferentiated proliferative multipotent cells called a blastema (Akimenko et al., 2003). Additionally, it appears that many of the mechanisms that regulate regeneration of any one tissue are common throughout regeneration elsewhere in the organism. Comparing the gene expression profiles from regenerating zebrafish retina, caudal fin, and heart muscle, for example, revealed multiple candidate genes that are shared among the tissues, suggesting these factors are potentially important for epimorphic regeneration (Qin et al., 2009). Identifying the molecular events that permit tissue regeneration is critical for understanding regulation of regenerative pathways in the fish and using them for potential human therapies (Poss, 2010).

A notable breakthrough for regenerative science came in 2009 when researchers at the Salk Institute for Biological Studies studied zebrafish fin regeneration and discovered enzymes involved in histone demethylation, an essential cellular pathway that initiates limb regeneration by switching cells at the amputation site from an inactive to an active state (Stewart et al., 2009). Kdm6b.1, an enzyme found exclusively in cells undergoing regeneration, was particularly important as its knockdown halted fin regeneration completely. Identifying the targets of enzymes like kdm6b.1 during regeneration may bring scientists closer to understanding the molecular events that coordinate the spectacular regrowth of injured tissue and figuring out ways to apply such information to human injuries and diseases.

## STEM CELL TRANSPLANTATION

Distinguishing transplanted donor cells from endogenous host cells, as well as being able to trace donor cells over time, are necessary requirements of stem cell transplantation assays. The availability of transgenic lines capable of fluorescently labeling specific cells and tissues, in conjunction with the optical transparency of embryos and the adult double mutant, *casper*, make the zebrafish a valuable organism for studying transplantation events *in vivo*. Once transplanted into the host animal, transgenic donor cells can be easily identified and traced using a simple fluorescent dissection scope or confocal microscopy, if a higher resolution is desired. A system that has benefited greatly from transplantation experiments in the zebrafish relates again to blood.

From work done with hematopoietic cell transplantation (HCT) in the mouse, much has been understood regarding the regulatory factors of HSC biology. Applying what is known about HCT from the mouse to the zebrafish model, which allows for easy visualization as well as larger-scaled experimentation, has already revealed novel mechanisms underlying HSC regulation and has the potential to be applied to other stem cell systems as well.

To date, transplantation of HSCs into zebrafish has been successfully performed between the following donor to host developmental stages: embryo to embryo (Bertrand et al., 2008), adult to embryo (Traver et al., 2003), and adult to adult (Traver et al., 2004; White et al., 2008).

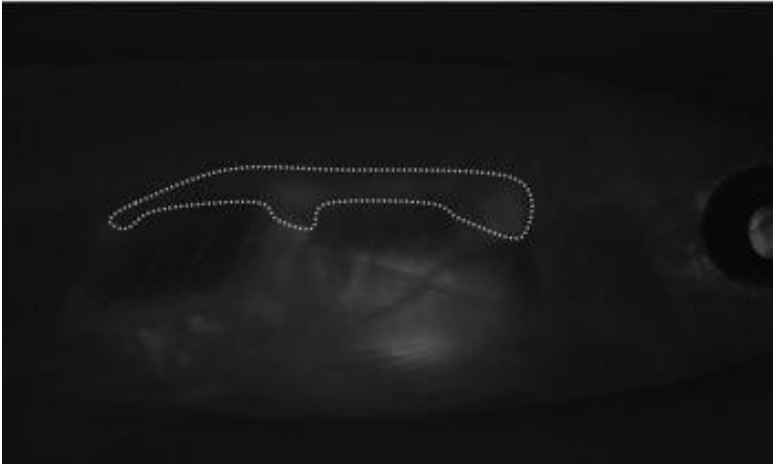
In adult zebrafish, the general protocol for HSC zebrafish transplantation begins with sub-lethal (25Gy) irradiation of host fish two days before transplantation. Optimization of irradiation dose, including split dose treatments, may decrease the death rate (Taylor and Zon, 2009). This irradiation will destroy a portion of the host's innate hematopoietic system and temporarily open up HSC niches for acceptance of transplanted HSCs. Transplantation into an embryonic host, on the other hand, implies that the mutant fish harbors some class of hematopoietic deficiency already present in the embryo and thus does not require irradiation.

Whether transplanting in adults or embryos, the next step requires the donor cells to be harvested from transgenic fish that are fluorescent for the cells of interest, in this case HSCs. The donor fish are sacrificed and whole kidney marrow (WKM), the site considered to be the organ of definitive hematopoiesis in the zebrafish, is collected via dissection. Occasionally, WKM is mixed with wild-type peripheral blood that will serve as a "carrier" if the protocol for specific sorted cells requires. Once the cells are isolated, they are transplanted into an adult host by either intracardiac (LeBlanc et al., 2007) or retro-orbital (Pugach et al., 2009) injection. If the recipient is

an embryo, transplantation is achieved by injection into the sinus venosus of 48 hpf fish.

After transplantation, *in vivo* imaging of the fluorescent donor cells *in vivo* is possible through stereomicroscopy of the transparent embryos or *casper* adult hosts. A more quantifiable readout of the transplantation's success can be achieved via flow cytometric analysis of the donor WKM, where fluorescent, transplanted cells are isolated and counted after the period of engraftment is over. Detailed protocols for dissecting kidney marrow cells, transplantation and post-transplantation analysis can be found in (LeBlanc et al., 2007) and (Pugach et al., 2009).

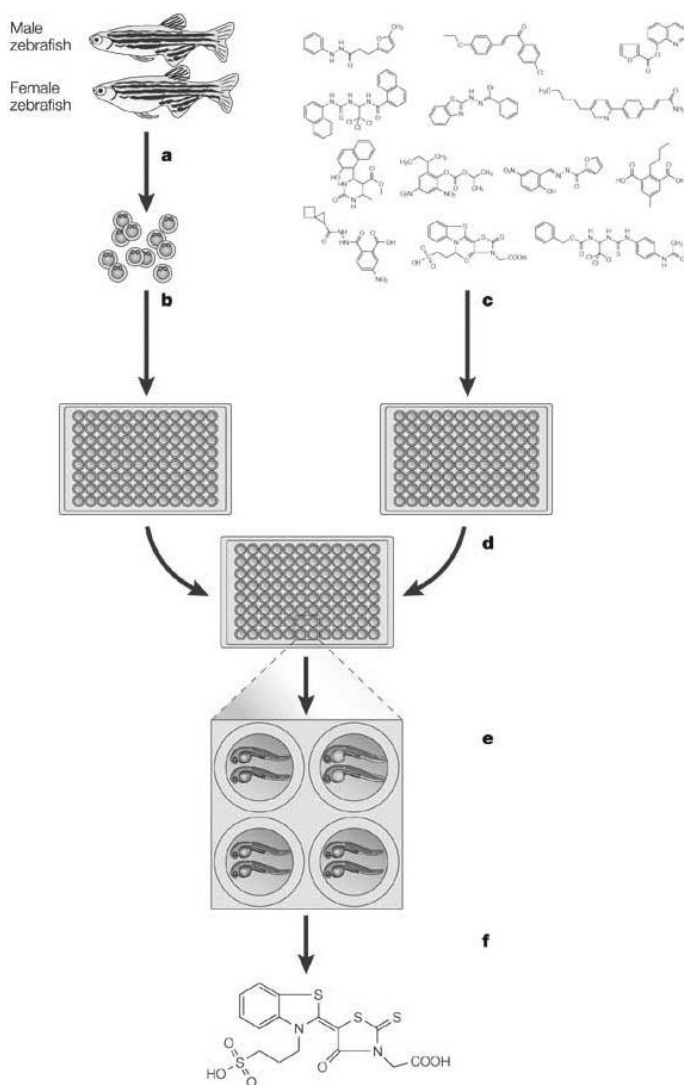
In addition to HSCs, transplantation of stem cells into zebrafish has been performed with neural crest (Schilling et al., 1996) and spermatogonial (Nobrega et al., 2010) stem cells, in addition to being a promising model for the transplantation of cancer stem cells (White et al., 2008).



**Figure 4. fluorescent cells transplanted into irradiated adult casper recipient.** image of whole kidney marrow cells, harvested from a tg( $\beta$ -actin:gfp) donor zebrafish, repopulating the kidney of an irradiated adult casper four weeks after their retro-orbital injection into the recipient fish (from pugach et al., 2009).

## CHEMICAL SCREENS

As the intrinsic mechanisms and niche interactions that regulate the fate of stem cells become better understood, knowledge of chemicals that modulate such processes becomes increasingly important for stem cell biology and therapeutic developments. While *in vitro* chemical screens have been successful in identifying and modulating pathways involved in self-renewal, differentiation, reprogramming, and therapeutic targeting of stem



**Figure 5. chemical screening to identify small molecule modulators.** a) adult zebrafish are mated to produce embryos used in the chemical screening. b) embryos are arrayed in 96-well plates. c) a small-molecule library containing potentially active compounds is synthesized or acquired. d) small molecules are added to wells either singularly or in combinations. e) after chemical incubation, the phenotypic effects of the small molecules on the zebrafish are determined visually or through an automated read-out. f) wells containing interesting phenotypes are referenced in the library to reveal the identity of the biologically active chemical (from zon and peterson, 2005).

cells (Xu et al., 2008), the potential of zebrafish for enhancing and furthering these and other pursuits via *in vivo* analysis is rapidly being realized. The same biological traits and genetic tools that make zebrafish amenable to a host of other stem cell applications are equally advantageous for chemical screening. The ability to conduct cost-effective, high-throughput screens in a transparent organism makes the phenotypic readout of a chemical's influence on endogenous stem cell behavior particularly useful. Additionally, the benefit of screening small molecules in a whole organism system inherently biases the screen toward compounds that are more likely to be cell permeable, less toxic, and possess acceptable pharmacokinetic and pharmacodynamic profiles, thereby increasing their likelihood of successful clinical translation (MacRae and Peterson, 2003).

Like the genetic screens mentioned earlier in this chapter, zebrafish small molecule screens are a classic means of disrupting biological processes and identifying genes or gene products involved in molecular functions *in vivo* (Peterson and Fishman, 2004). Unlike their genetic complement, however, chemical screens offer three distinct advantages: first, they can be performed with small molecule libraries made up of compounds with known biological function, allowing the rapid elucidation of involved pathways; second, chemical treatment can occur at any development time point, allowing for the study of latent effects of genes during the organism's growth; lastly, chemical dosage can be controlled in order to achieve tissue specificity (Zon and Peterson, 2005). Detailed instructions for designing zebrafish chemical screens can be found in (Peterson and Fishman, 2011).

Once the chemicals are applied, the readout of such screening can be conducted in a number of different ways (Tan and Zon, 2011). Scoring for specific developmental morphology changes in the zebrafish allows for detection of chemicals that influence a tissue specific disease or pathway of interest. Conversely, scoring nonspecific developmental morphology changes in the chemically treated fish broadens this approach by allowing any phenotypic change in the fish to be considered for further study. These two scoring methods are based on phenotypic changes detected and characterized by eye. Behavioral scoring adds an additional method but may prove less useful for stem cell research. The last scoring method, however, has historically proven the most important for advancing stem cell research. This is sorting by cell state, wherein a secondary assay is performed after chemical screening to identify modulations not evident to the naked eye, such as changes in mRNA expression levels, protein phosphorylation, and cell mitotic state.

Three types of secondary assays have been applied to the zebrafish system for analysis after the chemical treatment: *in situ* hybridization (North et al., 2007; Paik et al., 2010; Yeh et al., 2009), immunohistochemistry (Murphey et al., 2006; Stern et al., 2005a; Stern et al., 2005b), and fluorescent



protein reporter expression assays (Molina et al., 2009). Using these methods to detect previously unforeseen changes has illuminated numerous influences of small molecules on the organism as a whole.

One such screen that used the cell state sorting approach majorly advanced the fields of zebrafish chemical screening and stem cell translational research. By screening roughly 2,500 biologically active compounds for modulators of HSC formation and homeostasis, researchers discovered that chemicals interacting with the prostaglandin pathway were able to increase or decrease definitive HSCs in the site of embryonic hematopoiesis (North et al., 2007). Specifically, chemicals that enhanced prostaglandin E2 (PGE2) synthesis increased HSC numbers, as determined by in situ hybridization of HSC markers *runx1* and *cmyb* after the chemical application, while those that blocked prostaglandin synthesis decreased stem cell number in the zebrafish.

The results of this screen led to examination of the homing, survival, and proliferation of murine HSC engraftment when cells were exposed to PGE2 prior to transplantation (Hoggatt et al., 2009). Results of this treatment resulted in a four-fold increase in HSCs up to 20 weeks after transplantation, which strongly suggests that short-term PGE2 exposure leads to a long-term repopulation advantage of transplanted whole bone. Because of the data gathered from the chemical screen in zebrafish and subsequent investigations in mice, clinical trials are currently underway to test PGE2's ability to enhance HSC engraftment following cord blood transplantation in humans. For more comprehensive detailing of PGE2's development from zebrafish to clinic, please refer to a recent review by (Durand and Zon, 2010). PGE2 is just one example of how a chemical screen in the zebrafish can have significant implications for stem cell biology and human therapeutics.

## **CANCER STEM CELLS**

The diverse ways cancer can be studied in zebrafish are evident in the many recent reviews that detail the types of cancers currently modeled in zebrafish, the use of zebrafish in xenotransplantation of cancer cells, and the use of the zebrafish embryo to dissect the role of oncogenes and the biochemical signals activated in different cancers (Amatruda and Patton, 2008a; Amatruda and Patton, 2008b; Feitsma and Cuppen, 2008; Marques et al., 2009; Payne and Look, 2009; Stoletov and Klemke, 2008; Taylor and Zon, 2009). Thanks to advances in reverse genetic techniques, the ability to quickly generate stable transgenic or mutant zebrafish lines has led to the development of a wide variety of gene-based cancer models that have made zebrafish a major model for cancer research (Liu and Leach, 2011).

Despite 300 millions years of evolutionary divergence between fish and human, many important genes and pathways involved in human cancer formation are highly conserved in the fish. Because these cancer models are genetically and histologically similar to their mammalian counterparts, the zebrafish's unique morphological traits that make it amenable to *in vivo* analysis are now being used to study invasion, metastasis, angiogenesis, and cancer stem cell renewal in the live, whole organism setting.

Given the current inability to prospectively isolate cancer stem cells, limiting dilution transplantation experiments have been the most successful in establishing and estimating the number of cancer stem cells within a tumor (Tan et al., 2006). The zebrafish has been used in such assays with great success, as the proliferative capacity, self-renewal, and minimum number of tumor cells required for engraftment has been well characterized in the fish model. A protocol that describes the techniques for isolation and transplantation of tumor tissue in zebrafish can be found in (Dovey and Zon, 2009).

Using fluorescent activated cell sorting, cell transplantation, and limiting dilution analysis, Langenau et al. were able to identify the cancer stem cell behind zebrafish embryonal rhabdomyosarcoma (ERMS) (Langenau et al., 2007). They first created a robust transgenic model of RAS-induced ERMS that was morphologically similar to and expressed clinical diagnostic markers of human RMS and used this model to identify the serially transplantable cancer stem cell (Langenau et al., 2007).

As evidence continues to show that many pathways classically associated with cancer may also regulate normal stem cell development (as initially reviewed in (Reya et al., 2001), testing the cancer stem cell theory will require complex model organisms and advanced imaging techniques to help substantiate the existence of these rare cells. While the mouse has historically been the standard model, the many advantages of zebrafish screening, imaging, and transplantation discussed earlier in this chapter will continue to propel research forward.

## CONCLUSIONS

Given the many biological and morphological characteristics that allow for high-throughput, optically clear, rapidly-paced development, the zebrafish has established itself as a premier model for *in vivo* stem cell research for labs of all sizes and budgets. Early screening in this vertebrate model revealed thousands of mutants that continue to assist in the quest for understanding organogenesis and disease while technological advances have given scientists the capability to easily perform a broad range of genomic manipulations. Such advantages have led to groundbreaking imaging and analysis of stem cell migration, fate mapping, lineage

tracing, niche interaction, and regeneration. The zebrafish has become an increasingly important model for stem cell transplantation and chemical screening, in addition to work involving the isolation and characterization of cancer stem cells.

As screening techniques continue to become more sophisticated, imaging techniques more precise, and genomic alterations more controlled, the zebrafish holds great promise for advancing the understanding of stem cell self-renewal and differentiation on a molecular level as well as helping to define how homing and engraftment of stem cells can be further optimized for use in the clinical setting.

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## ABBREVIATIONS

|         |                                                 |
|---------|-------------------------------------------------|
| hpf     | hours post-fertilization                        |
| ENU     | ethyl nitrosourea                               |
| AP      | antero-posterior                                |
| Hox     | homeobox genes                                  |
| TILLING | Targeted Induced Local Lesions in Genomes       |
| SNPs    | single nucleotide polymorphisms                 |
| Zfn     | zinc finger nuclease                            |
| NHEJ    | non-homologous end joining                      |
| TALENs  | transcription activator-like effector nucleases |
| SSRs    | site-specific recombinases                      |
| DA      | dorsal aorta                                    |
| PBI     | posterior blood island                          |
| WISH    | whole-mount in situ hybridization               |
| WKM     | whole kidney marrow                             |

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# Deer Antler Stem Cells—New Aspects and Findings

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## SUMMARY

The annual regeneration of deer antlers is a unique developmental event in mammals, which as a rule possess only a very limited capacity to regenerate lost appendages. Studying antler regeneration can therefore provide a deeper insight into the mechanisms that prevent limb regeneration in humans and other mammals. With regard to tissue engineering and potential medical treatments, such studies may possibly even show ways how to overcome these limitations. First of all, we investigated the regenerative capacity of long-term cultivated cell populations derived from deer antler tissue. The results showed that primary antler cell cultures can be kept alive for more than 27 months without visible signs of apoptosis. Without artificial scaffolds, long-term cultures begin to form complex structures which give the impression that antlerogenic cells per se have the ability to build up a “trabecular” structure resembling a spongiosa. Traditionally, antler regeneration has been characterized as a process

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*List of abbreviation after the text.*

involving the formation of a blastema from de-differentiated cells. However, more recently it has been hypothesized that antler regeneration is a stem cell-based process. We were able to demonstrate the presence of cells positive for the mesenchymal stem cell marker STRO-1 in different areas of the pedicle and regenerating antler of fallow deer (*Dama dama*). After isolation and cultivation for extended periods of time, we found out that STRO-1<sup>+</sup> antlerogenic stem cells are able to differentiate along different lineages. Based on these results, it is assumed that the annual initiation of antler regeneration depends on the periodic activation of a stem cell niche mainly located in the pedicle periosteum. Furthermore, we demonstrated the ability of cultured STRO-1<sup>+</sup> deer antlerogenic mesenchymal stem cells (DaMSCs) to transfer the transcription factor Oct4 as a key regulator of pluripotency via direct cell-to-cell connection to recipient cells. While artificial induction of pluripotency using transcription factors is possible in mammalian cell culture, it remains unknown whether a potential natural transfer mechanism might be of functional relevance *in vivo*. With regard to the regeneration of deer antlers a transfer of pluripotency factors from resident stem cell niche cells located in the pedicle periosteum to surrounding differentiated non-niche cells could recruit more stem cells to start rapid tissue regeneration. All things considered, our recent results provide a new approach to explain the rapid annual antler growth.

## INTRODUCTION

In the course of evolution, higher organisms have lost the ability to regenerate injured or amputated parts of their bodies. Deer antlers are the only mammalian structures that regenerate completely every year. As cranial appendages, they develop on top of permanent processes (pedicles) present at the frontal bone of the skull. In the deer family (*Cervidae*), antlers are present in all male individuals. The sole exception are reindeer (*Rangifer tarandus*), in which both sexes carry antlers (Goss, 1983; Lincoln, 1992). Antlers consist of hard tissue similar to the bony structure of the human skeleton. The antlers of adult red deer (*Cervus elaphus*) achieve up to 15 kg weight and the annual antler regrowth represents one of the fastest growth rates of hard tissue observed in mammals. Some deer species exhibit extremely fast antler growth rates (up to 1–2 cm/day) leading to complete antler development from preosseous tissues to bone in an adult individual within 120–150 days (Goss, 1983). Therefore, antlers provide a unique natural model to study different aspects of rapid bone regeneration including skin and even nerve regeneration (Nieto-Diaz et al., 2012). Many of the growth regulatory controls and cell mechanisms involved in antler bone formation are identical or at least very similar to the bone formation process that occurs in humans. Since antlers are male secondary sexual

characteristics, the annual antler growth is mainly influenced by sex hormones and other endocrine factors (Bubenik, 1983, 1990; Goss, 1968; Rolf and Fischer, 1990, 1996; Suttie et al., 1998). For years researchers investigated whether hormones and/or factors, usually known to stimulate bone and cartilage growth (e.g., insulin-like growth factors, growth hormone) are also involved in annual antler growth, either alone or in combination with sex steroids (Elliott et al., 1996; Goss, 1968; Li et al., 2003; Suttie and Fennessy, 1992). In the last twenty years, this field of research was expanded by *in vitro* studies investigating the influence of such factors on the growth of cells derived from the proliferation zone of regenerating antlers (Gray et al., 1992; Price and Allen, 2004; Price et al., 1994; Rolf et al., 2006; Sadighi et al., 1994; Sadighi et al., 2001).

In contrast to mammals, lower vertebrates have a striking capacity to regenerate complex structures. Some amphibians are able to replace lost limbs completely and mysteriously; the healing process of this nature doesn't leave a scar. The epimorphic regeneration involves progenitor cells created through reprogramming of differentiated cells or through the activation of resident stem cells. Exploring the mechanisms of antler regeneration may provide crucial insights to better understand why mammals are unable to regenerate amputated limbs and, with regard to medical treatments, might even provide information that helps to overcome this inability some day. One of the most prominent researchers in the field of antler regeneration, Richard J. Goss, recognized these opportunities very clearly and must be credited for linking the study of antler regeneration to regeneration biology in general (Goss, 1980, 1983, 1984, 1995). The source of the cells that give rise to the regenerating antler has been a matter of controversy. Since 1942, deer researchers suggested that these cells originate from the pedicle dermis (Goss, 1972; Goss, 1984; Wislocki, 1942). Recently, most antler researchers consider the periosteum of the pedicle to be the source of the cells forming the regenerating antler (Kierdorf, 1992, 2001; Kierdorf et al., 1994; Kierdorf et al., 2003; Li et al., 2012; Li et al., 2007a; Li et al., 2005; Li et al., 2007b). The pedicle periosteum is a derivative of the antlerogenic periosteum that builds up the pedicle and the first antler (Goss and Powel, 1985; Hartwig, 1974; Kierdorf, 2002). The antlerogenic periosteum is discussed to be a piece of postnatal retained embryonic tissue (Li and Suttie, 2001).

Against this background, interest in the regenerative capacity of *in vitro* cultured antler tissue and the underlying molecular mechanisms moved centre stage (Faucheux et al., 2001; Gray et al., 1992; Lord et al., 2007; Mount et al., 2006; Price and Allen, 2004; Price and Faucheux, 2001; Rolf et al., 2006; Roubin and Gosh, 2001). It has been hypothesized that antler regeneration is a stem cell based process (Kierdorf et al., 2007; Kierdorf et al., 2003; Kierdorf, 2002; Li et al., 2005; Li et al., 2007b). According to this view, stem cells located in the pedicle periosteum give rise to progenitor cells of

different lineages, such as chondrocytes and osteoprogenitors (Kierdorf et al., 2003). However, until the year 2006 when our research group reported for the first time on isolated and cultivated stem cells derived from the deer antler/pedicle (Napp, 2006; Rolf, 2006), direct evidence for the existence of stem cells in the pedicle periosteum and the growing antler was lacking. As part of different research projects on deer antler regeneration, we searched for the presence of cells positive for known stem cell markers in pedicles and growing antlers of fallow deer. We investigated their localization within the pedicles and regenerating antler tissue and characterized an important stem cell population positive for the mesenchymal stem cell marker Stro-1 (Rolf et al., 2008). We found evidence that Stro-1<sup>+</sup> cells isolated from the growth zones of regenerating fallow deer antlers as well as the pedicle periosteum are able to differentiate *in vitro* along the osteogenic, chondrogenic and even adipogenic lineages. These results supported the view that the annual process of antler regeneration might depend on the periodic activation of mesenchymal progenitor cells located in a pedicle periosteum stem cell niche.

## **STRUCTURE FORMING CAPACITY OF DEER ANTLER TISSUE**

### ***In vitro* Investigations**

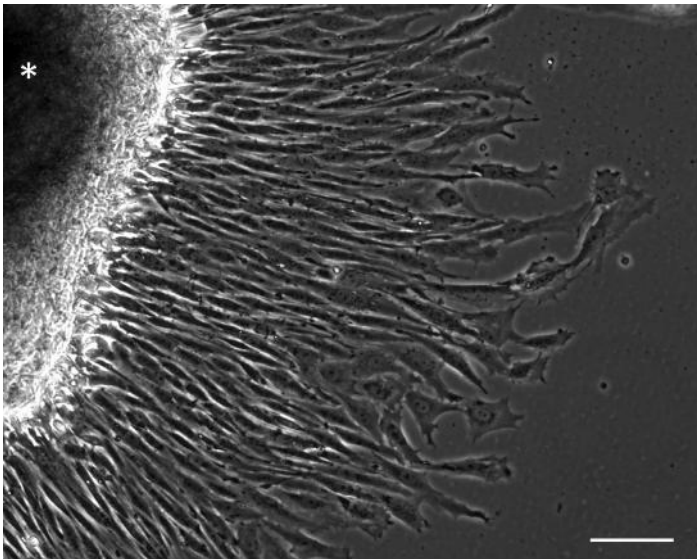
Interestingly, the annual process of antler regeneration starts before the deer cast their previous antlers. Once a year, the resting progenitor cells become powered by as yet unknown factors and the “activated” pedicle tissue is visible to the naked eye about 2–3 weeks prior to antler casting (Figure 1).

A number of researchers in the field of antler research investigated the regenerative capacity of deer antler tissue using *in vivo* methods like transplantation of antlerogenic periosteum (Gao et al., 2010; Kierdorf and Kierdorf, 2000; Li et al., 2001; Li and Suttie, 2001) or pedicle periosteum deletion (Li et al., 2007a; Li and Suttie, 1994). Recently, cells derived from growing deer antlers were also used in xenograft approaches (Cegielski et al., 2008; Cegielski et al., 2010a).

Similar to other colleagues (Faucheux et al., 2001; Price et al., 1994), our group also started cell culture experiments with tissue derived from regenerating deer antler and pedicle periosteum. In 2006, we reported on the structure forming capacity of long-term cultivated cell populations (Rolf et al., 2006). When tissue pieces from biopsy samples taken during antler regeneration were seeded into Petri dishes, a large number of single cells with greatly varying morphology started to grow out almost immediately from these tissue pieces followed by increasing cell proliferation (Figure 2).



**Figure 1.** Adult fallow deer about 2 weeks prior to antler casting. The swelling of the pedicle tissue below the “coronet” becomes macroscopic visible (detail, arrowheads). This point to the beginning of the annual antler regeneration based on activated progenitor cells located in the pedicle periosteum.



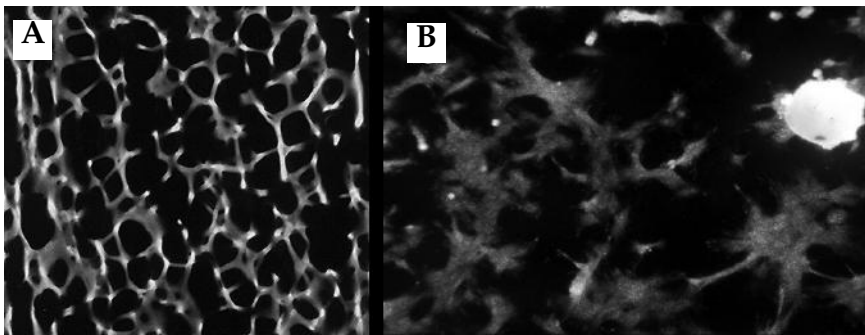
**Figure 2.** Antlerogenic cells growing out of a piece of antler tissue. Day seven of cell culture, asterisk = tissue piece, scale bar = 100  $\mu\text{m}$ .



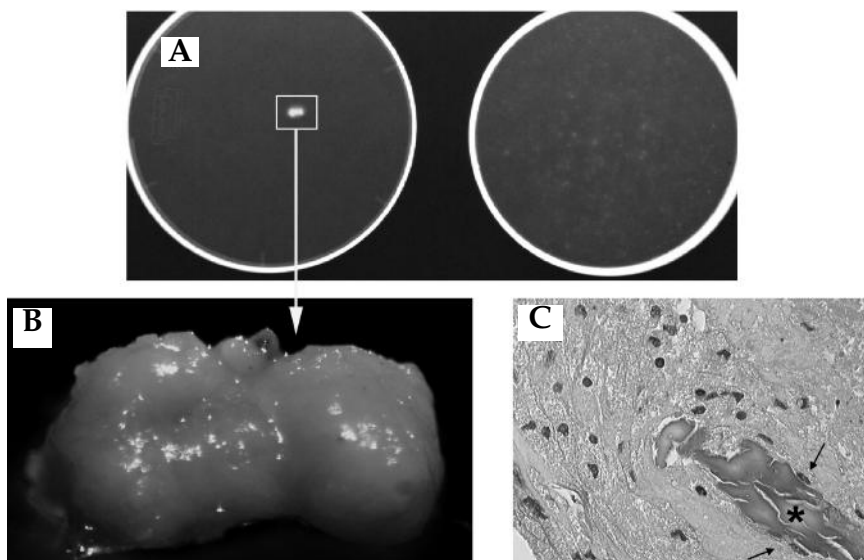
Since these cultures contained at least 3–4 different cell populations which were not yet fully characterized, we called them primary “mixed” cultures. As we previously described (Rolf et al., 1999), such mixed cultures are able to form complex structures if cells of primary cultures or first passages are used. In general, cultures of antlerogenic cells began to form cell accumulations and more complex structures from culture day 15 onwards. During 30 to 40 days of culture, these structures showed an increasing level of complexity resulting in trabecular-like structures. In addition, mineral deposition in the extracellular matrix of trabecular structures produced *in vitro* by “mixed” deer antler cell cultures could be observed. Compared to microradiographs of the spongiosa area of hard antlers, the complex structures of antlerogenic cells growing in long-term cultures give the impression that antlerogenic cells are building up a “trabecular” structure, resembling the development of spongiosa (Figure 3).

An increasing number of cell accumulations (nodules) developed after several months of cultivation and the mineralized structures and nodules appeared also in microradiographs (Figure 4a). In these cases some nodules reached sizes of up to 0.8 cm x 1 cm without the use of artificial scaffolds (Figure 4b). Histological examination of paraffin embedded nodules revealed partly organized structures with some mineralized areas resembling bone trabecula surrounded by cells which appeared to be resting osteoblasts (Figure 4c).

In the context of our long-term culture experiments with primary antler cell cultures as well as their first passages, we found out that antlerogenic tissue can be cultivated for more than 27 months without cell-passaging and without microscopically visible signs of apoptosis.



**Figure 3. Spongiosa of the deer antler—*in vivo* vs. *in vitro*** (A) Part of a longitudinal section of a hard antler; microradiograph of the spongiosa area ( $\times 2.5$ ); (B) Long-term culture of primary antlerogenic cells showing an organised «trabecular» structure ( $\times 10$ ). Taken from Rolf et al., 2006.



**Figure 4. Structures developed by long-term cultures.** (A) Microradiographs of petri dishes containing long-term cultures; right side = structures with mineralized areas (*same petri dish as shown in Fig. 2b*), left side = big nodule; (B) «bone» nodule formed in a culture dish by long term cultivated antlerogenic cells; size: 0.8 × 1.0 cm; (C) Histological section of a nodule containing a mineralized trabeculae with osteoblasts along its surface, asterisk = bone trabeculae, arrows = osteoblasts, HE - staining, (×100). Taken from Rolf et al., 2006.

A well-known deer researcher, G. A. Bubenik, reported that unlike the skeletal bone cells (which cease to divide after only a few generations) the preosseous tissues of the growing antler (such as the reserve mesenchyme, prechondroblasts, and chondroblasts) are able to grow in culture for several weeks without any sign of weakening mitotic activity (Bubenik, 1990). On this account, he concluded that the growing antler bone exhibits the properties of embryonic or neoplastic tissues, the latter till then being the classic material for carrying out tissue culture studies.

### Localization and Characterization of Stro-1<sup>+</sup> Deer Antlerogenic Mesenchymal Stem Cells (DaMSCs)

Based on the discussions that antler regeneration might be triggered by stem cells, we carried out FACS analyses of primary tissue cultures derived from different locations of the primary and regenerating antler as well as primary cultures derived from the pedicle periosteum of fallow deer. The analyses revealed, for example, that primary cultures derived from the pedicle periosteum contain STRO-1<sup>+</sup>, CD271<sup>+</sup> and CD 133<sup>+</sup> cell populations that are negative for CD34, a marker for hematopoietic progenitors (Rolf et

al., 2008). Therefore, such cells can be defined as mesenchymal progenitor cells. Based on these results, we obtained first evidence that the annual antler regeneration represents a stem cell-based process.

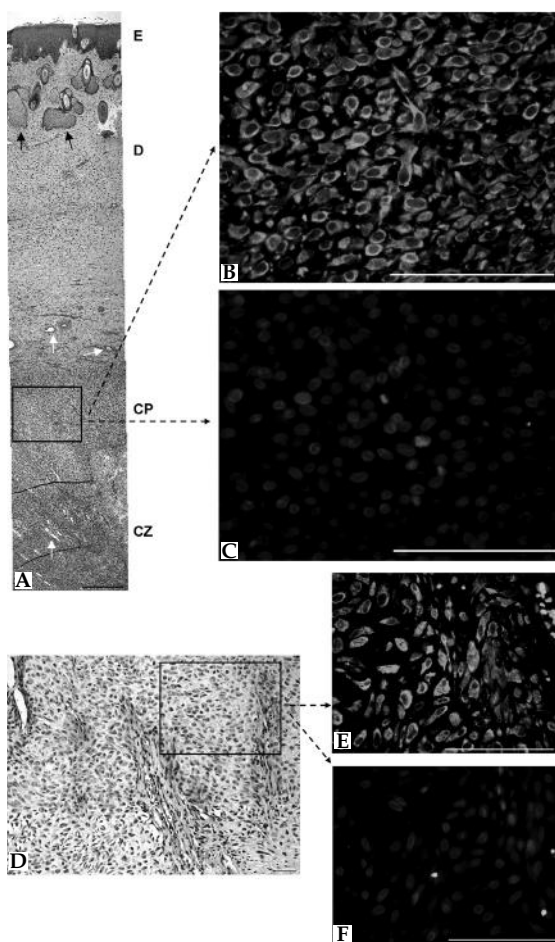
Since the Stro-1<sup>+</sup> cell population is attributed to contain osteogenic precursors (Gronthos et al., 1994) or even to be multipotential progenitors (Dennis et al., 2002) we searched for Stro-1<sup>+</sup> DaMSCs within pedicles and regenerating antlers using Stro-1 antibodies qualified for immunohistochemical investigations (Simmons and Torok-Storb, 1991).

In the regenerating antler, a high density of Stro-1<sup>+</sup> DaMSCs was found in the cambial layer of the perichondrium (Figure 5a-c) and within the chondrogenic growth zone (Figure 5d-f) present at the tips of the main beam and the antler tines.

Stro-1<sup>+</sup> DaMSCs were also present in different tissues of the pedicle (Figure 6a-c). Located between thick collagen fibers, such cells could be observed within the reticular layer of the dermis (Figure 6a, d-f). In addition, vascular associated Stro-1<sup>+</sup> cells were located in the subcutaneous tissue (Figure 6a, g, h). Corresponding to the situation in the regenerating antler, we found a high density of Stro-1<sup>+</sup> DaMSCs in the cambial layer of the pedicle periosteum (Figure 6a, l, m).

We used magnetic cell sorting to separate Stro-1<sup>+</sup> DaMSCs from primary mixed cultures and we found up to 13% Stro-1<sup>+</sup> cells in cultures derived from the chondrogenic growth zone of antlers of adult fallow deer (Rolf et al., 2008). In primary cultures derived from the pedicle periosteum of yearling fallow bucks we detected highest numbers of Stro-1<sup>+</sup> DaMSCs (about 17 %). The results of RT-PCR analyses showed that Stro-1<sup>+</sup> DaMSCs did not express key markers of the osteogenic (Cbfa1, osteocalcin) or chondrogenic (chondroadherin) lineages when cultured in standard DMEM containing 10 % fetal calf serum (Figure 7a). Only a weak expression of collagen I suggested that a few cells were already differentiated. Contrary to Stro-1<sup>+</sup> DaMSCs the Stro-1 negative antler cell populations exhibited marked expressions of the above key markers indicating the presence of cells belonging to osteogenic and chondrogenic lineages. Some examples of the typical morphology of isolated Stro-1<sup>+</sup> DaMSCs are shown in Figure 7b-e. Occasionally, "atypical" cells with three nuclei (Figure 7d, e) could be observed (Rolf et al., 2008), a phenomenon that to our knowledge has not previously been reported for stem cells in culture.

The most important finding of the study described above was the proof of Stro-1<sup>+</sup> stem cells located in different areas within the primary and regenerating antler as well as in the pedicle of fallow deer. As expected, we found peak numbers of Stro-1<sup>+</sup> DaMSCs in primary cultures derived from pedicle periosteum. These results are consistent with the hypothesis that the regenerating antler is build up by the progeny of mesenchymal stem cells located in the cambial layer of the pedicle periosteum (Kierdorf et al., 2007;



**Figure 5. Stro-1<sup>+</sup> DaMSCs in the cambial layer of the perichondrium and the cartilaginous zone of an antler.** Paraffin embedded biopsy samples of a velvet antler from a 4 year-old fallow buck (*Dama dama*); samples were taken 46 days after onset of regeneration. **(A)** Cross section of brow tine about 1 cm below the tip, overview, **(E)** epidermis, **(D)** dermis, **(CP)** cambial layer of the perichondrium, **(CZ)** cartilaginous zone, white arrows = vessels, black arrows = sebaceous glands; HE-staining, scale bar: 500 µm. **(B)** STRO-1<sup>+</sup> DaMSCs in the cambial layer of the perichondrium [STRO-1 antibody combined with an anti-mouse IgM secondary antibody conjugated with fluorescence dye (FITC), nuclei counter-stained with Hoechst 33342], scale bar: 100 µm. **(C)** Negative control, cambial layer of the perichondrium, same staining as **(b)** without STRO-1 antibody, scale bar: 100 µm, identical exposure times for pictures **(b)** and **(c)**. **(D)** Cross section of part of a main beam, cartilaginous zone, HE-staining, scale bar: 100 µm. **(E)** STRO-1<sup>+</sup> DaMSCs within the cartilaginous zone [same staining as **(b)**], scale bar: 100 µm. **(F)** Negative control, comparable area of the cartilaginous zone, same staining as **(e)** without STRO-1 antibody, scale bar: 100 µm, identical exposure times for pictures **(e)** and **(f)**. Taken from Rolf et al., 2008.

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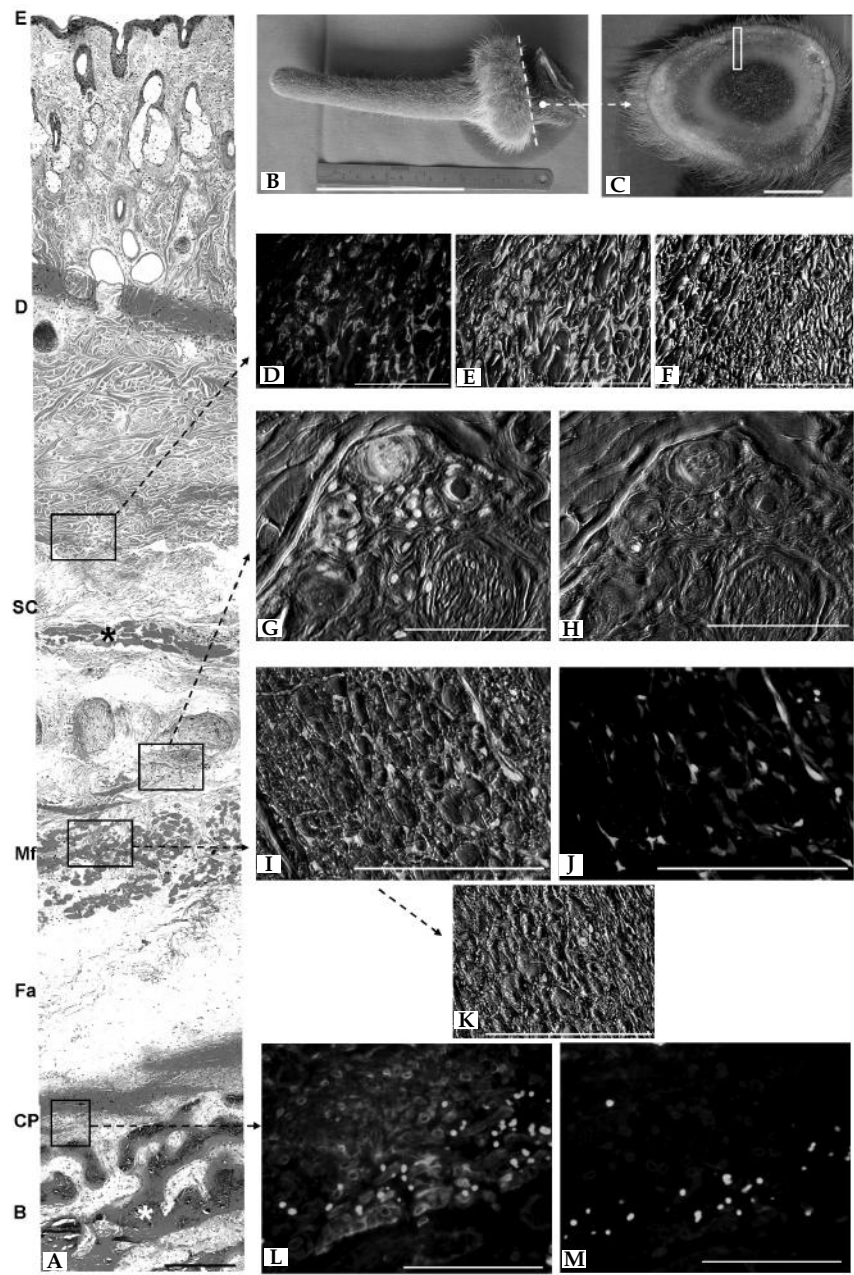
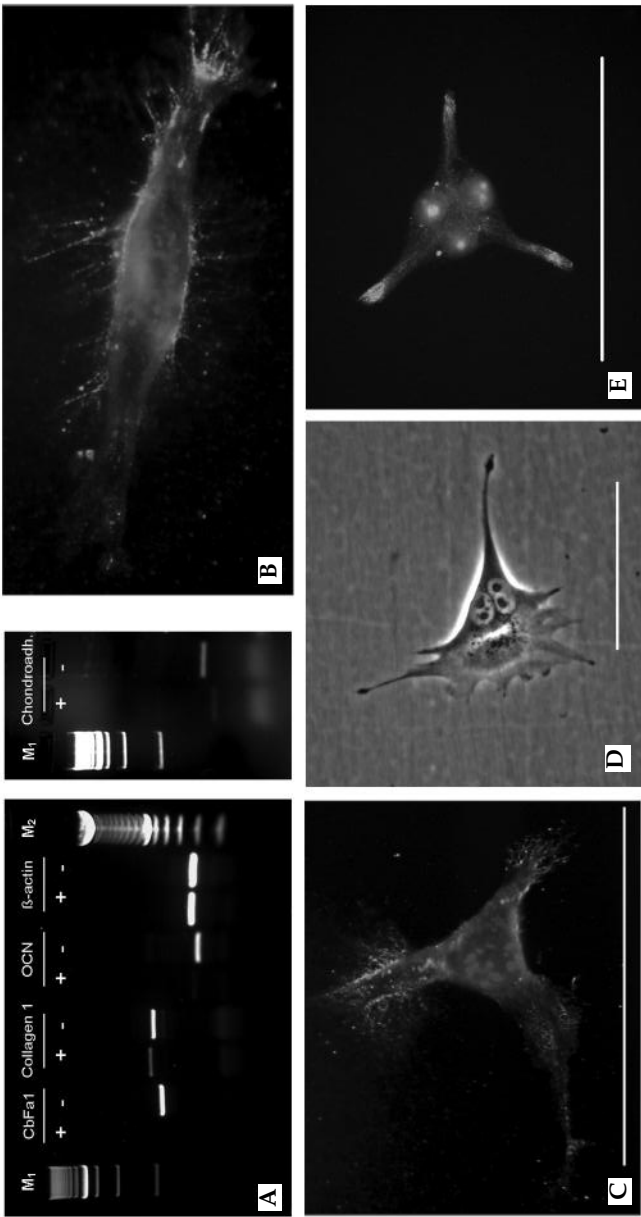


Figure 6. contd....

Kierdorf et al., 2003; Kierdorf, 2002; Li et al., 2005; Li et al., 2007b). Recently, it has been shown that in an adult individual stem cell populations exist in “niches”—specific anatomical locations that regulate how the stem cells participate in tissue generation, maintenance and repair (Jones and Wagers, 2008; Li and Xie, 2005; Moore and Lemischka, 2006; Scadden, 2006; Yin and Li, 2006). We assume that such a stem cell niche is located in the cambial layer of the pedicle periosteum and that the regeneration of antlers is dependent on the periodic activation of these stem cells. Our studies showed that *in vitro*, the differentiation potential of Stro-1<sup>+</sup> DaMSCs is not restricted to the osteogenic and chondrogenic lineages (Napp, 2006; Rolf et al., 2008). In addition to their ability to differentiate *in vitro* into cell types that naturally occur in growing antlers (e.g., osteoblasts), under appropriate culture conditions, Stro-1<sup>+</sup> DaMSCs were also able to differentiate into adipocytes. Contrary to long bones, adipogenesis does not occur in regenerating antlers. Therefore, we conclude that Stro-1<sup>+</sup> DaMSCs in deer antlers and pedicles represent a population of at least bi- or tripotent mesenchymal progenitor cells. Our results are in accordance with the findings that adipogenesis can be induced in cultured donor cell lines derived from antlerogenic periosteum of male red deer (*Cervus elaphus*) (Berg et al., 2007). Our results provide evidence for the contribution of stem cells in the process of antler regeneration. We suggest that the development of primary antlers and the yearly replacement of antlers in adult deer are both stem cell dependent

**Figure 6. Stro-1<sup>+</sup> DaMSCs in different areas of the pedicle.** (A) Methylmetacrylate embedded sample of the pedicle shown in (b) and (c); cross-section, overview, HE-staining. (E) epidermis, (D) dermis, (SC) subcutaneous tissue with superficial muscle (asterisk), (Mf) Part of the frontoscutular muscle, (Fa) fascia, (CP) cambial layer of the periosteum, (B) pedicle bone; white asterisk = bony trabeculae, scale bar: 500  $\mu$ m. (B) Left pedicle and primary velvet antler of a 1 year-old fallow buck (*Dama dama*), the antler was cut below the coronet (dashed line) to obtain a cross-section of the distal pedicle, scale bar: 10 cm. (C) Cross-section of the distal pedicle shown in (b); white rectangle marks the area shown in (a); scale bar: 1 cm. For all pictures (d-m). (D and E) Stro-1<sup>+</sup> DaMSCs within the reticular layer of the dermis, located between thick collagen fibres; (D) Stro-1<sup>+</sup> fluorescence, same area as (e); (e) Fluorescence combined with varel-contrast picture; (F) Negative control; similar area as shown in (e); the small green dots are erythrocytes marked by the fluorescence dyes; identical exposure times for pictures (e) and (f), scale bars: 100  $\mu$ m. (G) Vascular associated Stro-1<sup>+</sup> DaMSCs within the subcutaneous tissue, varel-contrast picture, scale bar: 100  $\mu$ m. (H) Negative control; same area as shown in (g); identical exposure times for pictures (g) and (h), varel-contrast picture, scale bar: 100  $\mu$ m. (I–K) Stro-1<sup>+</sup> DaMSCs between fibres of the frontoscutular muscle, scale bars: 100  $\mu$ m; (I) Fluorescence combined with varel-contrast picture; (J) Stro-1<sup>+</sup> fluorescence only, same area as (i); (K) Negative control, similar area as shown in (i); varel-contrast picture, identical exposure times for pictures (i) and (k); the bright green dots in picture (k) are erythrocytes marked by the fluorescence dyes. (L) Stro-1<sup>+</sup> DaMSCs within the cambial layer of the periosteum; scale bar: 100  $\mu$ m. (M) Negative control, similar area as shown in (l); scale bar: 100  $\mu$ m. Bright dots in pictures (l) and (m) are erythrocytes marked by the fluorescence dyes. Taken from Rolf et al., 2008.

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**Figure 7. Expression profiles and morphology of isolated STRO-1<sup>+</sup> DaMSCs.** (A) Expression profiles of Stro-1<sup>+</sup> negative versus Stro-1<sup>+</sup> cells. RT-PCR was used to detect the mRNA of specific markers for the osteogenic [Collagen 1, cbfa1, osteocalcin (OCN)] and the chondrogenic lineages (chondroadherin). Expression of deer  $\beta$ -actin was used for standardization. (+) = Stro-1<sup>+</sup> cells, (-) = Stro-1<sup>-</sup> cells, (M1) = Marker; 500 bp DNA ladder, (M2) = Marker; 100 bp DNA ladder. (B,C) Typical morphology of Stro-1<sup>+</sup> cells isolated from fallow deer antler cell cultures, scale bar: 100  $\mu$ m. (D,E) Stro-1<sup>+</sup> stem cells with three nuclei, (D) phase contrast picture; (E) same staining as shown in (b) and (c); scale bars: 100  $\mu$ m. Taken from Rolf et al., 2008.

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processes. In our opinion, antler regeneration involves the activation of stem cells located in a niche in the cambial layer of the pedicle periosteum. The presence of Stro-1<sup>+</sup> DaMSCs observed in different locations of primary and regenerating antlers suggests that these cells play an important role both for the formation of the interior component (e.g., bone and cartilage) as well as the external component (velvet skin) of the growing antler. In the case of antlers, extensive regeneration of a histological complex appendage in a postnatal mammal seems to be triggered by activation of resident stem cells located in different niches, e.g., Stro-1<sup>+</sup> DaMSCs located in the pedicle periosteum of the deer. This mode of differentiation is different from that occurring during limb regeneration in urodele amphibians and fin regeneration in teleost fish, which involve large-scale dedifferentiation and reprogramming of cells in the amputation stump. However, such mechanisms are under constant discussion (Akimenko et al., 2003; Carlson, 2005; Kragl et al., 2009; Morrison et al., 2006; Muneoka et al., 2008; Poss et al., 2003; Sanchez Alvarado, 2009). Based on the findings on antler regeneration, it could thus be speculated that induction of dedifferentiation in the stump tissue may also not be an indispensable step in promoting limb regeneration in mammals.

### **Intercellular Transfer of Pluripotency Factors between DaMSCs Located in the Pedicle Periosteal Niche**

Stem cells have the capacity for self-renewal and generation of differentiated cells to replenish lost or damaged tissue. In adult mammals, stem cell niches play an essential role in regulating these properties (Jones and Wagers, 2008; Li and Xie, 2005; Moore and Lemischka, 2006; Yin and Li, 2006). Based on the assumption that the annual antler regeneration is caused by pluripotent stem cells located in a pedicle periosteal niche, it remains unclear how a small population of persistent stem cells should be able to manage such rapid tissue regeneration.

In this context, we came upon new findings in the field of regenerative medicine. Recently, the availability of induced pluripotent stem cells by genetic transfer of pluripotency factors has enabled new approaches in stem cell research (Ho et al., 2011; Li et al., 2009; Vierbuchen et al., 2010; Yamanaka, 2007; Zhou et al., 2009). It has been established that mammalian somatic cells can be reprogrammed to pluripotent states by exogenous expression of the transcription factors Oct4, Klf4, Sox2 and c-Myc (Meissner et al., 2007; Park et al., 2008; Takahashi et al., 2007b; Takahashi and Yamanaka, 2006; Welstead et al., 2008). It has also been demonstrated that induced pluripotent stem cells could even be generated with fewer exogenous transcription factors supported by endogenous expression of reprogramming factors and/



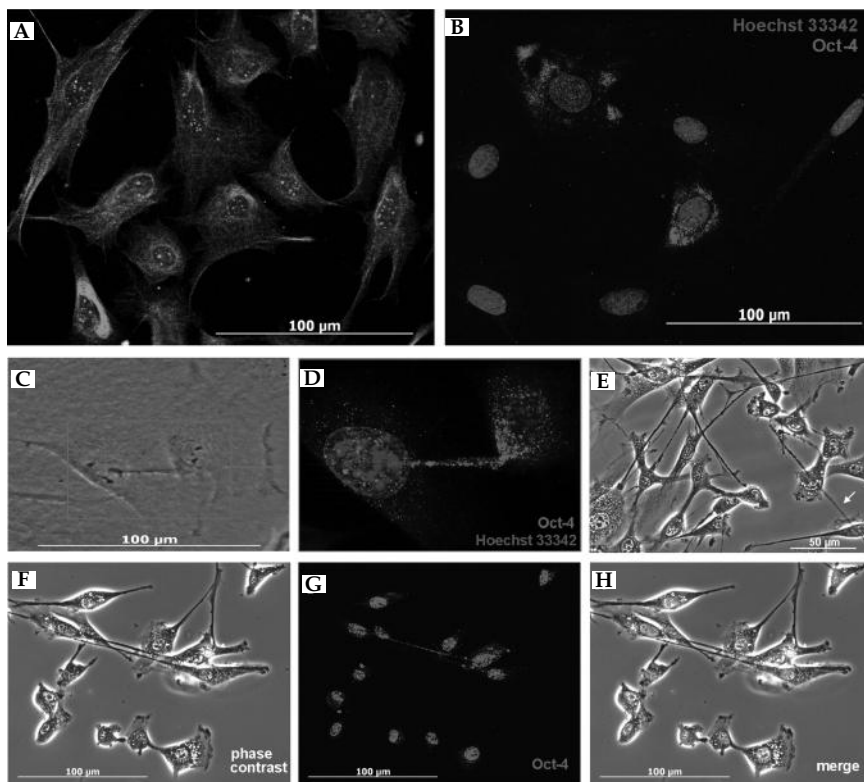
or synthetic small molecules (Kim et al., 2009b; Kim et al., 2008; Li et al., 2011; Tsai et al., 2010; Yuan et al., 2011). Within the scope of this research, the octamer-binding transcription factor 4 (Oct4, also known as Pou5f1), originally described as a marker of embryonic stem cells (Scholer et al., 1989), turned out to be a key factor in the induction of pluripotency. This was demonstrated by its ability to reprogram somatic cells, either alone (Kim et al., 2009a; Kim et al., 2009b; Takahashi et al., 2007a) or in concert with other factors (Kaji et al., 2009; Kim et al., 2008; Pan et al., 2010; Park et al., 2008; Tsai et al., 2010). Even in adult human stem cells, the expression of transcription factor Oct4 is thought to be vitally important (Ji et al., 2008; Pochampally et al., 2004; Tai et al., 2005; Tsai et al., 2004). It is also discussed whether Oct4 is a master regulatory gene in cell pluripotency and may serve as a pluripotency determinant in reprogramming (Li et al., 2011).

Thus, from the current point of view Oct4 as an essential reprogramming factor enables mature somatic cells to revert to an embryonic stem cell (ES)—like state, but it does not appear to be required for somatic stem cell self-renewal (Ralston and Rossant, 2010). It has been demonstrated that somatic cells can be fully reprogrammed into pluripotent cells by direct delivery of reprogramming proteins *in vitro* (Zhou et al., 2009). Based on the results that *in vitro* induction of pluripotency using defined factors is possible in mammalian cell culture, the question remains whether a similar mechanism might be of functional relevance *in vivo*. The idea of a possible *in vivo* induction of pluripotency requires a route of intercellular communication between stem cells of a niche and their immediate vicinity adapted for transmission of transcription factors like Oct4. The discovery of membrane nanotubes provided the basis for a cell-to-cell transport of intracellular material (Davis and Sowinski, 2008; Gerdes et al., 2007; Gerdes and Carvalho, 2008; Gurke et al., 2008a; Onfelt et al., 2005; Rustom et al., 2004). Zani and Edelman concluded that cellular bridges as putative routes for intercellular communication and cell migration provide the potential for directly affecting a greater area of the surrounding biological environment (Zani and Edelman, 2010). A transfer of cytoplasmic proteins between multiple cell types via transient membrane fusion was described by Niu et al. and they suggest that this phenomenon plays an important role in interactions between stem cells and adjacent somatic cells (Niu et al., 2009). In our opinion, with regard to antler regeneration, the described phenomena would be a prerequisite for the expansion of a DaMSC niche within solid tissue.

In the case of the annual regrowth of deer antlers, expansion of the DaMSC niche by induction of pluripotency in surrounding non-niche cells might be the key to understand by what means a small number of resident stem cells is able to accomplish the rapid tissue formation in regenerating antlers. For years, in our cell cultures derived from regenerating antlers

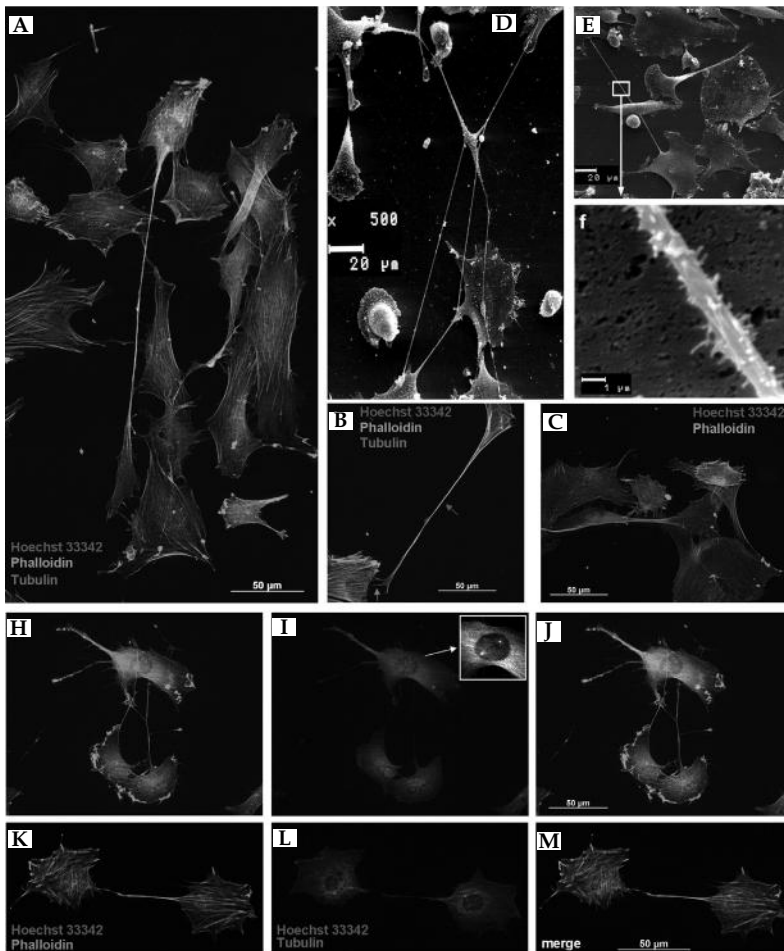
as well as the pedicle periost, we frequently observed long membrane extensions producing numerous cell-to-cell connections between antlerogenic cells. When we heard about the expression of Oct4 in human marrow stromal cells which was previously described (Pochampally et al., 2004; Rieckstina et al., 2009), we analyzed STRO-1<sup>+</sup> DaMSCs derived from the pedicle periosteum of fallow deer (*Dama dama*) and red deer (*Cervus elaphus*) for Oct4 expression. We found evidence that STRO-1<sup>+</sup> DaMSCs express the transcription factor Oct4 and that these Oct4 expression exhibited a time-dependent regulation (Rolf et al., 2012). While we found Oct4 in DaMSCs predominantly in the nucleus (Figure 8a), some cells showed enriched cytoplasmic staining in the perinuclear region as well as within long membrane extensions (Figure 8b, d, g). In our experiments, up to 50% of the investigated Stro-1<sup>+</sup> DaMSCs exhibited elevated Oct4 expression perinuclear and within cell-to-cell connections 2–4 days after initial cell sorting. Statistical analyses revealed that after 96 hours of cultivation the number of cells with cytoplasmic Oct4 expression decreased to about 11%. The amount of cells showing Oct4 expression within cell-to-cell connections decreased from 31% (24 hours after sorting) to 0.5% after 96 hours of cultivation. Oct4 expression within cytoplasm and cell-to-cell connections was no longer detectable after 144 hours of cultivation. We evaluated the distribution of Oct4 within immunostained Stro-1<sup>+</sup> DaMSCs during 0–144 hours of cultivation and we analyzed 165 microscopic images taken with high optical magnification. The data were based on more than 35 different Stro-1<sup>+</sup> DaMSC cultures (Rolf et al., 2012). Since we detected Oct4 expression only in Stro-1<sup>+</sup> sorted cells, we assume that sorting mimics a signal to activate Oct4 expression. In our opinion, this signal could either be the binding of the STRO-1 antibody or the loss of an inhibiting factor previously provided by cells of the primary mixed culture. This assumption needs to be clarified in future experiments.

Based on activated Oct4 genes in donor cells accompanied by a simultaneously Oct4 expression within the nuclei, the resultant cytoplasmic Oct4 synthesis is a prerequisite to enable transfer of Oct4 from donor cells to recipients. We never found Oct4 staining in cytoplasmic areas alone and therefore our results thoroughly point to a functional relevance of the Oct4 accumulation within cytoplasm and cell-to-cell connections. In our mixed cultures derived from antlerogenic tissues as well as in sorted DaMSC cultures, we regularly observed long-distance intercellular connections which span distances of 50 to >300  $\mu\text{m}$  (Figure 9). Frequently, these cell-to-cell connections have no contact to the substrate (Figure 8e, f; 9d-f). Staining against F-actin and  $\alpha$ -tubulin (Figure 9a-c and h-m) suggests that these membrane extensions are comparable to so-called tunneling nanotubes which were recently identified in a growing number of cell types like progenitor cells, immune cells, tumor cells or epithelial cells



**Figure 8. Intracellular distribution and intercellular transport of Oct4 in Stro-1<sup>+</sup> DaMSC cultures.** (A) DaMSCs 24 hours after cell sorting and subsequent cultivation in stem cell expansion medium. Antibodies against Stro-1 (pseudo-coloured green) and Oct4 (pseudo-coloured red) were used. Nuclei were counter-stained with Hoechst 33342 (blue). Cultured cells showing the surface marker Stro-1 and the transcription factor Oct4 is largely confined to the nuclei. (B) Single-staining with Oct4. Elevated Oct4 expression can be typically observed around 2–4 days after sorting and then about 10% of the cells exhibit intensive Oct4 staining with perinuclear localisation. (C) Phase-contrast picture of two Stro-1<sup>+</sup> cells showing a distinct cell-to-cell connection leading from one cell directly to the adjacent cell. The detail (D) demonstrates the same cells stained with Oct4 antibody (pseudo-coloured red). Oct4 immunostaining is visible perinuclear and within the cell-to-cell connection. One cell exhibits a higher Oct4 concentration which is enriched towards the membranous tube. (E) Phase contrast picture demonstrating that in the majority of cases the intercellular connections are not attached to the substrate. The white arrow point to a floating long-distance cell connection. (F) Phase-contrast picture of Stro-1<sup>+</sup> cells linked by thin cell connections. (G) Oct4 immunostaining (pseudo-coloured red) indicates the presence of Oct4 protein in the nuclei of the cells as well as inside the cell-to-cell connections. (H) Merged image of pictures (F) and (G). Partly taken from Rolf et al., 2012.

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**Figure 9.** Cytoskeletal elements of *Stro-1*<sup>+</sup> DaMSCs and long-distance cell-to-cell connections. (A,B) Different cell-to-cell connections between *Stro-1*<sup>+</sup> cells. Intercellular connections are able to bridge long distances even across neighbouring cells (Phalloidin/Tubulin staining, merged images). (B) Thick tubes (diameter >0.4 μm, example is marked by red arrow) contain F-actin and α-tubulin, whereas thin tubes (diameter <0.4 μm, example is marked by green arrow) contain only F-actin. (C) Negative control, staining without Tubulin antibody. (D–F) Scanning electron microscope pictures of a mixed culture of antlerogenic cells. Cells form long connections across neighbouring cells (D,E) and very high magnification (F) proves that the surface of tunneling nanotubes exhibit small appendages; long-distance connections are also possible between morphologically distinguishable cell types (D). (H–M) Multichannel pictures of Phalloidin (H,K) and Tubulin (I,L) stained DaMSCs demonstrate that their intercellular connections continuously consist of F-actin but only partially of microtubules. The visible spindle apparatus (I, detail enlargement) point to an initiating cell division and provides evidence that the used α-tubulin antibody is also efficient in DaMSCs. (J,M). Merged Images. Taken from Rolf et al., 2012.

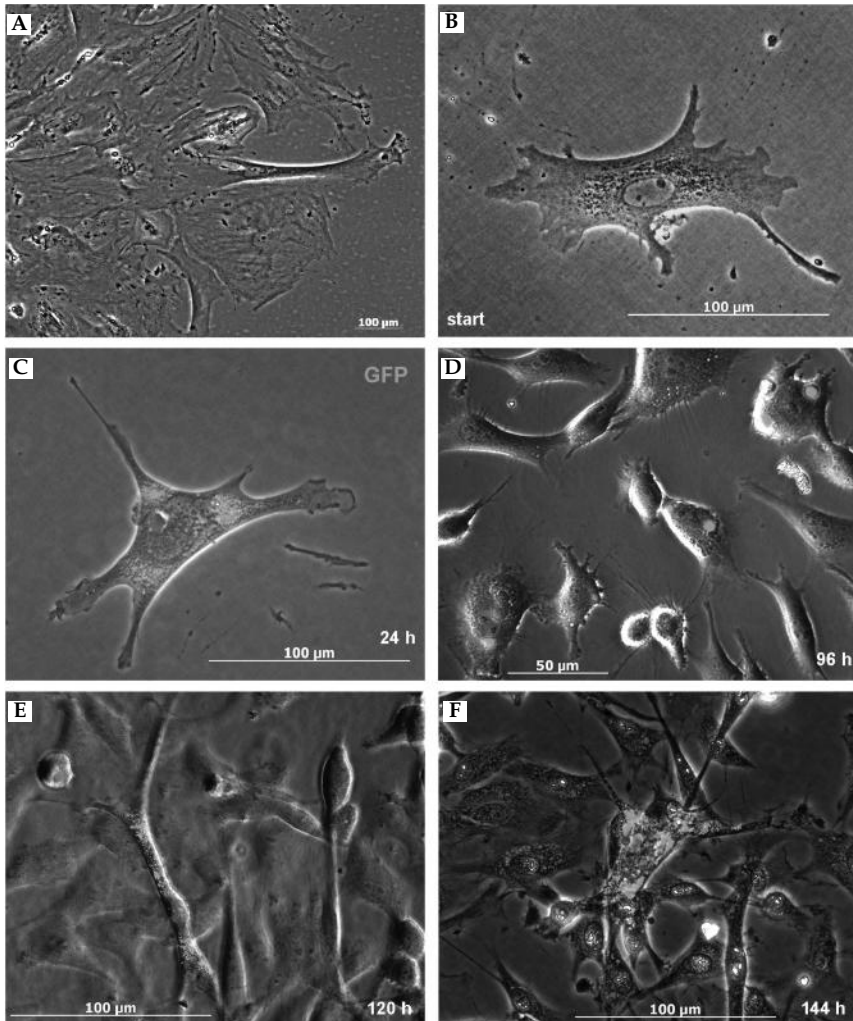
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(Davis and Sowinski, 2008; Domhan et al., 2011; Gerdes and Carvalho, 2008; Gurke et al., 2008a; Rustom et al., 2004). We also observed different types of nanotubular cell connections (Rolf et al., 2012) as previously described in the literature (Gerdes et al., 2007; Gerdes and Carvalho, 2008; Onfelt et al., 2006; Rustom, 2009; Rustom et al., 2004). Since tunneling nanotube like structures are known to transport cargo of various sizes ranging from proteins (Gerdes, 2009), over viral particles (Sowinski et al., 2008) to entire organelles (Gurke et al., 2008b), the growing number of reports on tunneling nanotube like structures point to an underlying general principle of cell-to-cell communication (Gerdes and Carvalho, 2008).

As we observed Oct4 staining within cell-to-cell connections between Stro-1<sup>+</sup> DaMSCs (Figure 8), we used a transfection approach with a plasmid encoding fluorescence labeled Oct4 to demonstrate transport of Oct4 within these tunneling nanotube like structures (Rolf et al., 2012). In transfected Stro-1<sup>+</sup> DaMSCs, the Oct4 fusion protein was transported unidirectionally (away from the cellular body) via cytoplasmic connections. The transport appears similar to mechanisms of intercellular communication described, e.g., for urothelial cell lines (Veranic et al., 2008). This indicates that a mechanism enabling transfer of transcription factors exists in DaMSCs.

### **Transfer of Transcription Factor Oct4 from Stro-1<sup>+</sup> DaMSCs to Indicator Cells**

Since we found evidence that Oct4 can be transported between DaMSCs along cell-to-cell connections, we wondered whether the transcription factor will be able to enter other cell types and then induce transcription in recipient cells. To demonstrate possible functional consequences of such a transfer we used the murine Oct4 reprogramming control cell line Stemgent<sup>TM</sup> Oct4-GFP MEF (Brambrink et al., 2008), in which GFP expression is under the control of an endogenous Oct4 promoter. We co-cultivated Oct4-GFP MEF cells together with Stro-1<sup>+</sup> DaMSCs and monitored for GFP expression (Rolf et al., 2012). In controls, neither indicator cells (Figure 10a, b), nor Stro-1<sup>+</sup> DaMSCs exhibited GFP expression when cultured alone. In MEF cells we detected GFP expression already 24 hours after co-cultivation with Stro-1<sup>+</sup> DaMSCs had started (Figure 10c). Within the following days, increasing intracellular GFP expression as well as increasing numbers of GFP<sup>+</sup> cells could be observed (Figure 10d-f). Although our co-cultures consisted of cell-types derived from two different animal species, increasing intercellular interactions could be observed (Figure 10d) and GFP<sup>+</sup> cells became well integrated into the forming multilayer (Figure 10e, f). Considering the recent literature, the combined use of murine and deer cells should not cause problems. Ho et al. described that the diversity of cell types and species that



**Figure 10. Mixed culture of Oct4-GFP mouse embryonic fibroblasts cells and Stro-1<sup>+</sup> DaMSCs.** (A) Colony of Oct4-GFP mouse embryonic fibroblasts cells without any GFP expression. (B) Individual Oct4-GFP mouse embryonic fibroblasts cell after 24 hours of pre-cultivation in stem cell expansion medium on the eve of co-cultivation. (C) The same Oct4-GFP mouse embryonic fibroblasts cell after 24 hours of co-cultivation with Stro-1<sup>+</sup> DaMSCs (interacting DaMSCs are outside of the display window). GFP expression (green) is visible within the cytoplasm. (D) Mixed culture after 96 hours of co-cultivation. More GFP<sup>+</sup> cells interacting with GFP2 cells are visible. (E) Cell with distinct GFP expression after 120 hours of co-cultivation. At that time GFP<sup>+</sup> cells interact continuously with GFP2 cells and are well integrated into the forming multilayer. (F) After 144 hours of co-cultivation some cells exhibit widely distributed intracellular GFP expression. (a,b,c,f = phase contrast pictures; d,e = varel contrast pictures; a–e = pictures of living cultures, f = fixed cells). Taken from Rolf et al., 2012.

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have been reprogrammed and the general applicability of Oct4, Sox2, c-Myc and Klf4 suggests a generic fashion in which these reprogramming factors act (Ho et al., 2011). They concluded that there probably is no cell type-specific barrier that cannot be overcome by the action of reprogramming factors leading to an evolutionary conserved pluripotency network.

Interestingly, these authors hypothesized that all somatic cells may be amenable to reprogramming, but more undifferentiated cells in the population have a higher probability to overcome reprogramming barriers. Another study about the reprogramming of human somatic cells which used various neonatal somatic cells supported this theory. This study revealed differences in reprogrammability between the investigated cell types (Maherali et al., 2008). The results also point to different kinetics of reprogramming, suggesting that some cell types might be more amenable to reprogramming than others. With regard to our co-culture experiments, the results left no doubt that Stro-1<sup>+</sup> DaMSCs released Oct4 directly via cell-to-cell connections. Possible alternatives, for instance a conceivable intercellular transfer of Oct4 via exosomes (Simons and Raposo, 2009) could be excluded (Rolf et al., 2012). There is evidence to suggest that Oct4-GFP MEF recipient cells had already received the transcription factor from Stro-1<sup>+</sup> DaMSCs after 24 hours of co-cultivation resulting in an initiating GFP expression. Considering recent studies, it seems unsurprising that the target cells in our experiments react immediately to the transferred Oct4. As described by Li et al., experiments with MEF cells suggested that after initiation, reprogramming is independent of continuous exogenous Oct4 expression (Li et al., 2011). Based on these findings it seems conceivable that very small amounts of Oct4 protein transported via TNTs are sufficient to initiate an immediate reaction in the recipient cell.

Recently, the expression of key transcription factors like Oct4 in antlerogenic tissue has been also confirmed by proteomic analyses (Li et al., 2012). Regarding our co-culture experiments, the onset of GFP expression from the endogenous Oct4 promoter represents an activation of this important pluripotency marker gene and may point to an initiation of reprogramming in the Oct4-GFP MEF indicator cell line. However, our experiments were only designed to prove a possible transfer of the transcription factor Oct4 from STRO-1<sup>+</sup> DaMSCs to potential recipients. Two fundamental questions remain: (1) whether Oct4 in this context acts alone or in concert with different co-factors and (2) whether the Oct4-dependent initiation of transcription in Oct4-GFP MEF cells in our co-cultures induces real reprogramming or mere proliferation.

## A NEW HYPOTHESIS TO EXPLAIN RAPID STEM CELL-BASED ANTLER REGENERATION

With regard to deer antler regeneration, we presume that a special situation exists within the deer's pedicle periosteum. In our hypothesis, a persistent stem cell niche mainly consisting of Stro-1<sup>+</sup> DaMSCs might be surrounded by more or less differentiated somatic cells amenable to the transcription factor network that regulates pluripotency. Once a year, this niche is powered by as yet unknown factors. The DaMSCs begin a process of self-renewal and simultaneously, a contingent of these cells starts to transfer Oct4 and/or other factors/co-factors to the surrounding environment to enable immediate and rapid tissue regeneration. In principle, the expression of Oct4 in Stro-1<sup>+</sup> deer antler stem cells suggests that at least some parts of the stem cells originating from the pedicle periosteum possess even more primordial characteristics and a potential beyond mesenchymal cell types. Above all, the functional effect of the Oct4 protein across species barriers observed in our co-cultures supports the theory of an evolutionary conserved pluripotency network. In our opinion, the direct intercellular transfer of transcription factors represents a basic principle that also remains in other mammalian stem cells. If this is appropriate, it might be fundamentally relevant to the interpretation of stem cell niche capabilities.

## CONCLUSION

There is still much to be learned about the similarities and differences of the mechanisms that prevent the regeneration of amputated limbs in mammals (including deer) and that allow and regulate the periodic regeneration of deer antlers. In regenerative medicine and "tissue engineering", an increasing number of stem cell approaches is used in treatment and repair of injured tissues and organs as well as in engineering tissue to be used in reconstructive surgery. Recently, a discussion about a potential application of antlerogenic stem cells has begun (Cegielski et al., 2010b). Without a doubt, the process of antler regeneration offers an interesting model to investigate the mechanisms which enable a relatively small population of stem cells located in a niche to accomplish rapid and complete tissue regeneration in an adult mammal. Understanding of the underlying biological processes will have important implications in biomedical and tissue research. The application of stem cells in regenerative medicine requires comprehension of stem cell differentiation and behavior both *in vitro* and *in vivo*. Experiments with antlerogenic stem cells can make an important contribution to this research area. For example, the results of our studies indicate that not only



limited tissue regeneration, but also extensive appendage regeneration in a postnatal mammal can occur as a stem cell-based process. The observed transfer of transcription factors indicate a possible mechanism for the expansion of a resident stem cell niche by induction of pluripotency in surrounding non-niche cells via direct transfer of pluripotency factors through intercellular connections. Provided that this is a basic principle in mammalian stem cells, it offers new approaches in exploring regeneration procedures. A great challenge will be the exploration of factors regulating stem cell niche activities. In the case of deer antler regeneration, a precise, localized activation of the pedicle periosteal niche occurs once a year without any noticeable influence on farther tissue areas. To elucidate factors and mechanisms that enable the activation and/or switching off of stem cells located in a niche might be of fundamental importance for researchers working in the area of regenerative medicine.

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## ABBREVIATION

DaMSCs                      deer antlerogenic mesenchymal stem cells

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# Genetic Manipulations of Pluripotent Stem Cells

*Konstantinos Anastasiadis*

## SUMMARY

Genetic manipulations of mammalian cells have become fundamental for our efforts to understand gene function. Embryonic stem cells (ESC) have an advantage over other cell types for performing mutagenesis studies. One reason is the easiness for introducing genetic modifications in the ESC genome. The efficiency of homologous recombination in mouse ESC is high, thus enabling the generation of gene targeted alleles with desired modifications. The second reason is that ES cells are pluripotent and can be differentiated *in vitro* into all three germ layers or in the case of mESC can give rise to an entire organism after injection into preimplantation embryos. The study of the phenotype caused by a mutation is not only limited to undifferentiated ESCs but also to a variety of different cell types that can be generated downstream. In this review we will discuss the two main avenues for manipulating the ESC genome: transgenesis and gene targeting. Further we will discuss new technological developments, which enable the performance of functional studies with ES cells.



## INTRODUCTION

There are different reasons for conducting genetic manipulations in pluripotent stem cells: a) gene function studies by overexpressing a wild type (wt) copy or a dominant-negative form of a cDNA in a constitutive or inducible manner b) gene function studies by conditional mutagenesis (knock out) or inducible knock-down c) production of reporter cell lines for analyzing lineage commitment d) establishment of cell lines for directing differentiation e) insertion of protein tags for proteomic analysis f) generation of disease models by gene targeting or chromosome engineering g) correction of mutations by gene targeting. For some of the applications described above, one can use either a gene targeting approach or transgenesis that leads to random integration. For loss of function studies one needs either to disrupt the gene of interest or interfere with its expression level. Gene disruption is usually accomplished by gene targeting but in order to study the function of a gene in diploid cells, one has to disrupt both alleles, a process that is not easily achieved. Alternatively one can use RNAi (RNA interference) to downregulate the expression level of the gene. The disadvantage of RNAi is that the knock-down never leads to a complete loss of function and it can also cause off-target effects. Nevertheless, is a very powerful method that can be used for genome wide screenings of different biological pathways in various cell types that are transfectable with RNAi reagents such as esiRNA, siRNA or shRNA (Ding et al., 2012; Mohr et al., 2010). A successful screening depends on the availability of a reporter assay that serves as the read out of the investigated biological function. Furthermore because RNAi screens are usually transient and their effect last for a couple of days they can only be used with a reporter system that responds fast to the induced gene expression changes. Gene targeting can be also used to generate reporter lines, introduce protein tags or correct genetic mutations. For all those applications one can also use transgenes that will be integrated randomly into the genome. On the one hand gene targeting is the most elegant way to manipulate the genome but on the other hand is a laborious process and for some of the above applications a transgenesis approach may be more suitable for delivering the desired outcome. In addition to the issues we will discuss below, several reviews on mouse genome engineering provide details that due to space constraints are not described here (Adams and van der Weyden, 2008; Adamson et al., 2011; Glaser et al., 2005).

## TRANSGENESIS

By the term transgenesis we usually refer to the stable integration of foreign DNA randomly into the genome. The transfer of genetic material

is achieved by various transfection methods such as electroporation, lipofection, nucleofection, viral transduction, microinjection, etc. Different types of vectors can be used for transgenesis, such as plasmids, cosmids, fosmids, bacterial artificial chromosomes (BACs), viral-type vectors or transposons all with their advantages and disadvantages. There are also vectors, which do not integrate into the genome, such as episomal vectors or viral vectors like HSV (Herpes Simplex Virus) and AV (Adenovirus) (Howarth et al., 2010).

Typically, small-size vectors that contain regulatory elements (promoter, enhancer), the coding sequence of the gene of interest and polyadenylation sequences are used for transgenesis. In cases where selection for stable integration is required, a gene that confers resistance to an antibiotic (selection marker) is included into the same construct. The expression of the selection marker is driven either by a separate promoter or from the same promoter as the gene of interest using elements such as viral internal ribosomal entry site (IRES) or 2A peptides that allow bicistronic expression (Kim et al., 1992; Ryan et al., 1991). The main problems encountered with randomly integrated small-size vectors (plasmids or viral vectors) are ectopic expression and silencing. For plasmids this is due either to position effects exerted from neighboring elements in the genome or to integration as concatamers. To avoid integration as concatamers one can use viral vectors or transposons, which integrate as single copies. This mode of insertion doesn't exclude integration into different genomic sites. There are different types of viral vectors that were used for delivering foreign DNA, such as retroviral, adeno-associated (AAV), lentiviral (Howarth et al., 2010; Osten et al., 2007). Lentiviral vectors are the most widely used nowadays and can transduce both dividing and non-dividing cells (Sakuma et al., 2012). The disadvantage of lentiviral vectors is the limited size of transgene DNA or cargo (~10 kb) that can carry. Silencing of viral vectors in mouse and human ES cells is occurring more frequently with retroviral vectors (such as Moloney Leukaemia Virus) and less with lentiviral.

DNA transposons are mobile elements that in their original configuration contain a transposase flanked by inverted terminal repeats (ITRs) and operate by the "cut and paste" mechanism. The transposase binds to the ITRs and "cuts" the whole transposon from the genome and "pastes" it into a new site (Ivics et al., 2009). The use of transposons as gene vectors in mammalian cells started only recently with the isolation of Tol2 from Medaka fish, the isolation of piggyBac (PB) from the cabbage moth *Trichoplusia ni* (Ding et al., 2005) and the reactivation of Sleeping Beauty (SB) from the salmon genome (Ivics et al., 1997). For a better control over transposition reactions, the transposase can be expressed in a constitutive or inducible manner from a separate vector and the ITRs can be used to flank a cassette of interest (cargo) for delivery into the genome. Transposition

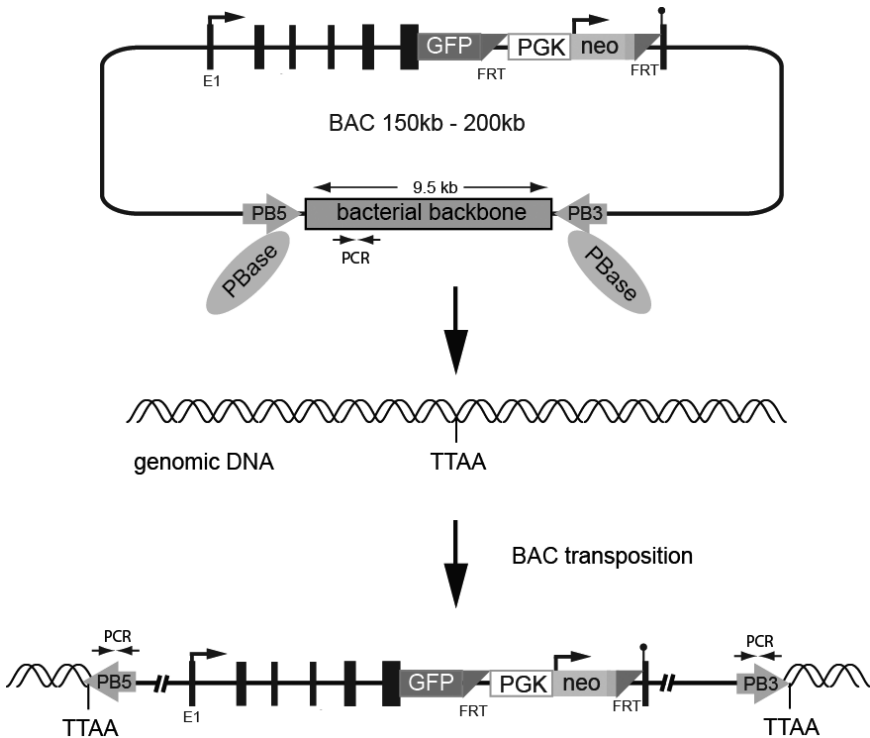
efficiency is limited by the size of the cargo. Recently, versions of the SB and PB transposases with an enhanced activity have been developed, that show transfection efficiencies similar to viral transduction (Cadinanos and Bradley, 2007; Mates et al., 2009; Yusa et al., 2011). Transposons can be also excised from the genome by re-expression of the transposase. The target site of the piggyBac transposon is the TTAA motif in the genome, which is duplicated at the site of integration. After excision there is only the genomic TTAA sequence left behind. Because of this property, the piggyBac transposon system has been used for generating induced pluripotent stem cells that were subsequently devoid of the reprogramming factors (Yusa et al., 2009).

Still, the problem of transgene silencing due to chromosomal position effects is not solved. Flanking the transgene with chromatin opening elements, scaffold/matrix-attachment regions (S/MARs) or insulator elements can prevent silencing (Chung et al., 1993; McKnight et al., 1992; Zhang et al., 2010). The ubiquitous chromatin opening element (UCOE) of the human *HNRPA2B1-CBX3* locus prevents silencing of lentiviral vectors that is mediated by DNA methylation (Zhang et al., 2010). On the other hand insulator elements can either function as a barrier to inhibit heterochromatin expansion or block the interaction of neighboring elements with the regulatory sequences of the transgene (Gaszner and Felsenfeld, 2006). The most commonly used element in mammalian cells is the chicken beta globin insulator, which combines both blocking and barrier properties and has been shown to work efficiently in ES cells and mice (Anastassiadis et al., 2010).

## **BAC TRANSGENESIS**

Bacterial Artificial Chromosomes are cloning vectors that can carry large DNA fragments (up to 350 kb). It is anticipated that BACs carry most if not all of the regulatory sequences of the gene of interest, therefore guarantying an expression pattern that resembles the endogenous gene. BACs can be easily modified by recombineering technology (homologous recombination in *E. coli*) in various ways including insertion of reporter fluorescent genes or protein tags, deletion of defined domains or introducing point mutations (Muyrers et al., 2000; Muyrers et al., 1999). BACs have been widely used for the generation of transgenic animals (Giraldo and Montoliu, 2001). Modified BACs can be transfected very efficiently into mouse ES cells by lipofection (Hofemeister et al., 2011). Transfection of BACs into human ES cells or other cell types by lipofection was suboptimal; therefore alternative transfection methods such as electroporation or nucleofection were applied (Placantonakis et al., 2009). The main problem with BAC transgenesis is how to guarantee that the BAC integrates intact. Electroporation and

nucleofection can break the BAC leading to integration of BAC fragments. A method that relies on BAC transposition has been shown to work very efficiently for mouse and human ES cells (Li et al., 2011; Rostovskaya et al., 2012). BACs that are previously modified to carry a fluorescent reporter gene and a selection marker are further retrofitted in the bacterial backbone with transposon inverted terminal repeats, which flank another antibiotic selection marker (e.g., ampicillin). The modified BACs are transfected by lipofection together with the transposase. If the BAC is integrated by a transposition mediated mechanism, then it should contain the inverted repeats and lose the selection marker that is positioned in-between (Figure 1). Screening for a transposition event is easily achieved by PCR and indeed all clones that showed a transpositional signature contained



**Figure 1.** Transposition mediated BAC transgenesis. The GFP is inserted before the stop codon of the gene of interest by recombineering technology in a BAC. The PGK-neo selection cassette is flanked by FRT sites and can be excised by Flp-mediated recombination. The inverted terminal repeats (ITRs) PB5 and PB3 of the piggyBac transposon are inserted left and right of the bacterial backbone. The piggyBac transposase (PBase) binds to the ITRs, cuts and separates the genomic fragment from the bacterial backbone and integrates the genomic fragment into a TTAA site in the genome, which is duplicated after insertion. Transposition mediated integration of the BAC can be monitored by PCR amplification of the two inverted repeats and lack of a product from sequences present in the bacterial backbone.

intact BACs integrated as single copies (Rostovskaya et al., 2012). Another advantage of this method is that the inverted repeats can serve as a DNA tag allowing the precise mapping of the integration site in the genome. In contrast to the wide held belief that transposons cannot carry large cargoes, Rostovskaya et al. showed that pieces of up to 200 kb can be inserted in the genome by transposition.

## **GENE TARGETING**

By gene targeting we refer to the insertion of an exogenous DNA fragment into a predetermined site into the genome. Gene targeting relies on homologous recombination, which happens with higher frequency in ES cells as compared to other somatic cell types.

## **TYPE OF TARGETING VECTORS**

Historically we can distinguish between two types of gene targeting vectors: Insertion and Replacement type vectors (Thomas and Capecchi, 1987). Insertion type vectors undergo a single reciprocal recombination event with the target sequence. Gene targeting efficiency is higher with insertion vectors but they lead to a duplication of the genomic target sequence (Hasty et al., 1991). Gene replacement vectors undergo two reciprocal homologous recombination events (Shigeoka et al., 2005) and became the vectors of choice for gene targeting. A classical replacement type vector contains a 5' homology arm, an antibiotic selection marker and a 3' homology arm. So far there are 4 antibiotic resistance genes used as positive selection markers in mouse ES cells: neomycin, hygromycin, puromycin and blasticidin. In addition to the positive selection marker, the insertion of a negative selection marker such as the Herpes Simplex Virus Thymidine Kinase (HSV TK) outside of one of the homology arms is used in combination with the appropriate drug (Gancyclovir or FIAU (1-(-2-deoxy-2-fluoro-1-b-D-arabinol-furanosyl)-5-iodouracil)) for increasing targeting efficiencies. Cells with randomly integrated targeting vectors that contain the HSV TK will die upon addition of FIAU, whereas in cells with correct targeted events the HSV TK will not get integrated and will survive under selection conditions. Negative selection is conferred also by the expression of the Diphtheria Toxin A (DTA) gene without the need of an additional drug. Selection takes around 10–15 days for the mESC and longer for hES cells and in some cases there is requirement for feeders that are resistant to the applied antibiotic. Selection without antibiotics can be achieved by replacing the selection markers with fluorescent proteins. Similar to the positive/negative principle, one fluorescent protein is used

for positive selection and the other is placed downstream of the homology arms and is used for negative selection using flow cytometry. Another advantage of the fluorescence based selection scheme is that it takes shorter time and thus it can be applied to cell types that are not easily to maintain in an undifferentiated state in culture such as haematopoietic stem cells (Hatada et al., 2005). In addition there are selection schemes that depend on the expression of the targeted locus. Disruption of the X-linked *Hprt* (hypoxanthine phosphoribosyltransferase) gene in male mouse and human pluripotent stem cells confers resistance to 6-TG (6-thioguanine) and sensitivity to HAT (Hypoxanthine, Aminopterin, Thymidine) medium, whereas repair of a mutated *Hprt* gene results to the opposite (Doetschman et al., 1987). Another example is the *PIG-A* gene that when disrupted in hES cells can confer resistance to aerolysin (Zou et al., 2009).

The design of the targeting vector also depends on the screening strategy and should be done in parallel. Screening for correct targeted events is usually done by Southern hybridization, using probes that anneal outside of the homology arm region (5' and 3' probe) and internal probes for excluding additional random integration events of the targeting vector. Screening by long range PCR using primers that anneal outside the homology arm and in the cassette is a faster method but Southern hybridization is the most reliable way to confirm targeting. Therefore and in order to allow convenient screening, the Southern probes should be tested before constructing the vector and the length of the homology arms should be adjusted accordingly.

The efficiency of gene targeting further depends on the length and isogenicity of the homology arms, the chromosomal position and expression level of the gene. Initially, replacement vectors were designed with a long homology arm on one side and a short homology arm (ca. 1 kb) on the other side to allow rapid screening of correct targeted events by conventional PCR. Increasing the length of homology arms results in higher targeting frequencies but on the other hand very long homology arms can be problematic for the screening of correct targeted events. If the gene is not expressed in ES cells then a constitutive promoter is used to drive the expression of the selection marker. The most often used constitutive promoters are phosphoglycerate kinase1 (PGK1) and beta actin. If the gene is expressed in ES cells then one can rely on the expression of the endogenous gene promoter to drive the expression of the selection marker. This requires the inclusion of a splice acceptor upstream of the selection marker and resembles the "gene trap" type of vectors. The decision between a promoterless or a promoter-driven selection marker type of vector depends on where to set the threshold of gene expression. Using as a reference the transferrin receptor (*Trfr*), a housekeeping gene that shows moderate expression, Friedel et al., showed

that all genes with expression levels above 5% of Trfr can be targeted by a promoterless vector (Friedel et al., 2005).

A classical question related to the efficiency of gene targeting is the following. What is more efficient: to insert an extra piece of DNA into the genome or to delete a genomic fragment? Using the human *HPRT* locus as the target sequence, Russell and Hirata (2008) showed that targeting efficiencies, measured by colony numbers resistant to 6-TG were higher when a piece of DNA such as a selection marker is inserted into the *HPRT* locus than with vectors which would result in deletions of the *HPRT* gene (Russell and Hirata, 2008).

## **GENE TARGETING WITH BAC VECTORS**

BACs can be easily modified using recombineering technology, which enables the insertion of selection cassettes into predefined positions of the BAC in *E. coli* (Muyrers et al., 1999). BACs in which a reporter gene and a selection marker replaced the whole gene or parts of it have been used for targeting mouse ES cells in a high throughput manner (Valenzuela et al., 2003). Because the large homology arms contained in the BAC do not allow screening for correct targeted events by classical methods such as Southern blot or long range PCR, a screening strategy that relies on quantitative PCR to detect loss of one wt allele has been developed. Targeting efficiency of BACs as compared to shorter targeting vectors was higher and this difference was more pronounced for loci with low targeting frequency. Also there was no difference in targeting frequencies between different mouse ES cell lines, meaning that the isogenicity issue is solved by the long stretch of homology provided in the BAC vector. BAC vectors have been also applied for targeting both alleles of the p53 (TP53, Tumor suppressor protein 53) and the ATM (Ataxia Telangiectasia Mutated) loci in human ES cells. Initial attempts to target the ATM gene with classical targeting vectors that contained homology arms of 2 kb and 9 kb long failed. Sequential targeting of both alleles of the ATM gene using a BAC vector with a 5' homology arm of more than 100 kb and a short 3' homology arm (6, 5 kb) for facilitating screening of the targeted events resulted in efficiencies around 20% (Song et al., 2010).

## **GENE TARGETING USING VIRAL VECTORS**

The use of viral vectors for gene targeting emerged mainly from the difficulty to introduce foreign DNA into human pluripotent cells. Adeno associated viral (AAV), Helper Dependent Adenoviral (HDAdVs), Integrase defective lentiviral and Baculoviral vectors have been used successfully for targeting

human pluripotent cells (Aizawa et al., 2012; Lei et al., 2011; Lombardo et al., 2007; Mitsui et al., 2009; Russell and Hirata, 1998). In AAV targeting vectors, the part that contains homologous sequences for the region to be targeted and a selection cassette is flanked by inverted terminal repeat sequences necessary for viral packaging. After packaging, the vector genome is single stranded. A drawback of AAV-vectors is their limited capacity to carry foreign DNA (around 5 kb), which can be a disadvantage if longer homology arms are needed for increasing the efficiency of homologous recombination. Another viral vector used for gene targeting in human and primate (*Cynomolgus* monkey) ES cells and in human iPS cells is the Helper-Dependent Adenoviral vector (Aizawa et al., 2012; Liu et al., 2011; Suzuki et al., 2008). Because all viral genes have been completely removed, HDAdVs are less cytotoxic than the parental ones and can also carry larger DNA fragments than AAV. This gives the possibility to build targeting constructs with longer homology arms or incorporate additional DNA elements into the same vector.

## **GENE TARGETING MEDIATED BY ENGINEERED NUCLEASES**

Engineered nucleases are designed enzymes that can introduce a double strand break into a specific genomic site. The induced DNA break can get repaired either by Non Homologous End Joining (NHEJ) or by homology driven repair if there are homologous sequences provided by a targeting construct. The double strand break is repaired by the Synthesis Dependent Strand Annealing mechanism using the donor DNA as a template at both sides (Moehle et al., 2007). But also a combination of repair mechanisms with homology repair from the one side and NHEJ from the other side has been reported (Lombardo et al., 2007). There are three kinds of engineered nucleases available in the toolbox: Zinc Finger Nucleases (ZFNs), Transcription Activator Like Effector Nucleases (TALENs) and CRISPR-Cas Nucleases.

### **ZINC FINGER NUCLEASES**

Zinc finger nucleases (ZFN) consist of a series of zinc finger domains designed to recognize a unique sequence in the genomic DNA fused to the type II endonuclease FokI that after dimerization can introduce a double strand break (Urnov et al., 2010). Zinc finger nucleases work as pairs, with each partner recognizing a different sequence upstream/sense and downstream/antisense orientation of the target cleavage site. The dimerization of the endonuclease domain occurs only if the two partners of the ZFN pair bind to the recognition sites. Each zinc finger protein (ZFP)



recognizes approx. 3 bp of target DNA so that an array of three ZFP per monomer can recognize a sequence that is at least 9 bp long (from each side of the cleavage site, 18 bp per pair). Usually the length of the homologous sequences in the donor vector is around 800 bp upstream and downstream of the ZFN target site. The length of homology can be reduced to 50 bp for each arm without altering the targeting efficiency, which is around 5–10% in the human erythroleukemia cell line K562 for the AAVS1 locus (PPP1R12C), the site of Adeno Associated Virus (AAV) integration in chromosome 19 (Orlando et al., 2010). Moreover, single strand DNA oligonucleotides with a total homology that can be shortened down to 40 bases can be used in combination with ZFNs for introducing point mutations or create genomic deletions of up to 100kb (Chen et al., 2011).

## **TRANSCRIPTION ACTIVATOR LIKE EFFECTORS NUCLEASES (TALENs)**

An alternative to Zinc Finger Nucleases is provided by the Transcription Activator Like Effectors Nucleases (TALENs). TAL effectors are DNA-binding proteins found in plant pathogenic bacteria such as *Xanthomonas*. They consist of a central domain that contains a variable number of tandem repeats (15, 5 to 19, 5) with each repeat consisting typically of 34 amino acids (Boch and Bonas, 2010). In each repeat the amino acids at position 12 and 13 are variable, the so-called repeat variable diresidues (RVDs) and determine the DNA binding specificity of the TALEs (Boch et al., 2009; Scholze and Boch, 2010). One TALE repeat corresponds to one bp in the target DNA, implying that TALEs consisting of 17, 5 repeats can bind a sequence that is 19 bp long (the N-terminal flanking segment or “repeat 0” binds a Thymine and the half repeat binds the last nucleotide). Because the DNA binding specificity is provided by amino acids 12 and 13 of the repeat, one can design repeats that recognize a specific nucleotide array. Recently fusion of TAL effectors with the FokI endonuclease have been tested in human embryonic and induced pluripotent stem cells with efficiencies comparable to zinc finger nucleases (Hockemeyer et al., 2011).

There are certain advantages for using zinc finger nuclease mediated targeting. The length of homology can be short, thus facilitating the generation of targeting constructs. The disadvantages are the unspecific binding of the ZFN and cleavage in other genomic regions, generating double strand breaks that are repaired by NHEJ and that are only identified by genome wide sequencing. To reduce off-target effects and error-prone NHEJ, a strategy that relies on the use of nickases has been developed. Nickases produce single-strand breaks, which are repaired by homologous recombination. Nickases have been generated by engineering the FokI

nuclease domain of ZFNs and have been successfully tested in genome editing assays, although the efficiency of homologous recombination was lower than with ZFNs (Kim et al., 2012). Another disadvantage is the expensive and complicated design of ZFNs (Perez-Pinera et al., 2012). The recent development of TALENs is a promising and safer alternative to the ZFNs. The recognition sequence of TALENs is more specific and is not influenced by the neighboring repeats such as in the ZFNs where the array of ZF proteins are interdependent for their binding specificity. TALENs cannot tolerate more than 3–4 mismatches in the target sequence, indicating that the off-target effects will be limited (Scholze and Boch, 2011). TALENs can be constructed efficiently in the lab using Golden Gate cloning method (Cermak et al., 2011) or in a high throughput manner using a method abbreviated as FLASH (Fast Ligation based Automatable High-throughput System) (Reyon et al., 2012).

## **CRISPR-Cas NUCLEASES**

Recently, the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR), which is originally a component of an RNA-based adaptive immune system found in bacteria, has been used for genetic manipulations in eukaryotic genomes (Wiedenheft et al., 2012; Wang et al., 2013). An engineered chimaeric RNA guides the CRISPR associated nuclease (Cas) to cleave a complementary DNA sequence in the genome. The CRISPR-Cas system has been used very efficiently to introduce double strand breaks simultaneously into 5 different genes in mESC (Wang et al., 2013) and it is easier to design compared to ZFNs and TALENs. Although those are great advantages, the drawback of the CRISPR-Cas system is its relaxed specificity (Fu et al., 2013).

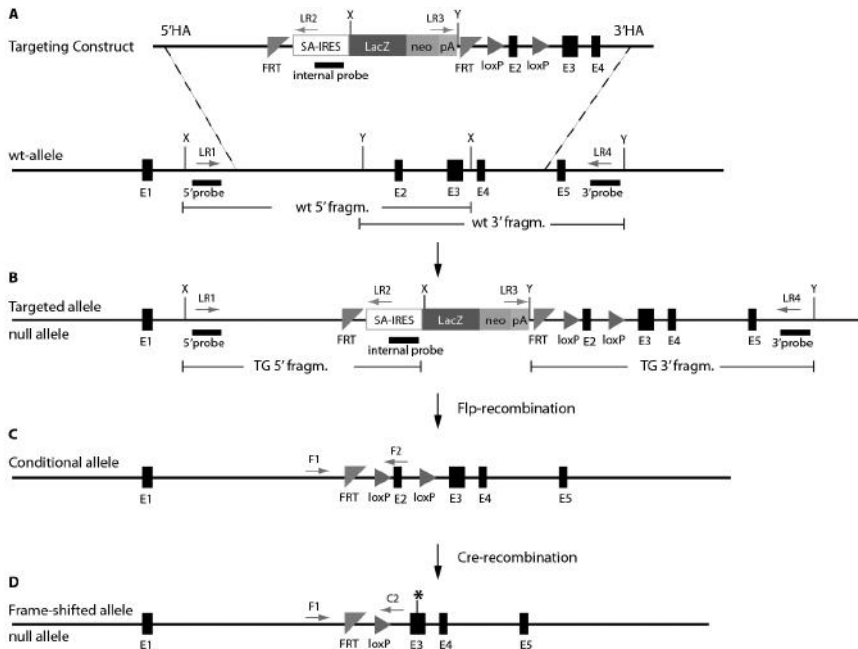
## **CONDITIONAL MUTAGENESIS**

Conditional mutagenesis refers to the temporal and/or spatial gene inactivation and is implemented for different reasons: a) to overcome putative embryonic lethality and thus allowing phenotypic analysis at later developmental stages or in the adult b) to restrict the analysis in specific cell-types or organs in cases where the loss of gene function causes pleiotropic effects. Conditional mutagenesis relies mainly on the use of site-specific recombinases. Today there are a number of site-specific recombination systems, which were successfully applied in mouse embryonic stem cells: Cre/loxP, Flp/FRT, Dre/rox, VCre/VloxP (Anastassiadis et al., 2009; Rodriguez et al., 2000; Sauer and Henderson, 1988; Suzuki and Nakayama, 2011). These enzymes catalyze 4 different reactions: excision/insertion,

inversion, translocation and cassette exchange depending on the orientation and position of their recognition sites in the genome. The target sites are composed of an asymmetric 8-bp core sequence, which determines the orientation of the site, flanked by 13-bp inverted repeats from each side. Each recombinase has its specific target sites (wt or homotypic) but also new target sites have emerged by introducing specific nucleotide substitutions either in the core sequence or in the inverted repeats (heterotypic sites). Reactions can occur either between two homotypic or two heterotypic sites but not between an homotypic and an heterotypic site. For more details on site-specific recombination action the reader is advised to read the following reviews (Branda and Dymecki, 2004; Grindley et al., 2006; Schnutgen et al., 2006).

Historically, site-specific recombination was initially applied for removing the selection cassette because the promoter elements that drive the selection marker can interfere with endogenous enhancer/promoter elements and alter the physiological expression of the gene or neighboring genes (Ohno et al., 1993). Having only one system available (Cre/loxP), the strategy was to flank the selection cassette with loxP sites and position an extra loxP site upstream or downstream of the selection cassette to flank (flox) the genomic sequence planned for deletion. This triple loxP strategy relied on inefficient Cre-recombination that could remove in ES cells or in mice only the selection cassette but not the flanked genomic region (Rajewsky et al., 1996), allowing the deletion of the genomic part of interest at a later time point using a more active Cre. Nowadays, by having more recombinases available, the selection cassette is flanked by FRT sites for FLP-mediated excision, whereas loxP sites for Cre-mediated recombination are reserved for flanking genomic regions (usually one or more exons).

For conditional mutagenesis, one is choosing a genomic region that upon deletion will result in loss of gene function. Ideally one would delete all exons, but this becomes a very difficult task for multi-exon genes that expand over a large genomic region. A general strategy that can be applied to most genes is to identify the most upstream exon(s) that when deleted result in a frame-shift and the appearance of a premature stop-codon. Subsequent activation of the non-sense mediated decay mechanism will degrade the mRNA. This so called critical exon(s) is flanked by site-specific recombination target sites (most of the cases loxP) and can be excised by Cre-recombination. A selection cassette that is flanked by FRT sites and contains a splice-acceptor, an antibiotic resistance gene (usually neo) and a reporter gene for expression such as LacZ followed by polyA is inserted in the intron preceding the critical Exon (Figure 2). In most of the cases the insertion of the selection cassette results in a chimaeric transcript composed



**Figure 2:** Conditional mutagenesis based on the “multi-purpose” allele strategy. **(A)** The targeting construct is composed of a splice-acceptor (SA)-IRES-LacZ-neo fusion followed by a polyadenylation signal (pA) and flanked by FRT sites (green triangles). The frameshifting exon 2 (E2) is flanked by loxP sites (red triangles). **(B)** In the targeted allele (KO first or null allele) transcription starts at exon 1 (E1) and is terminated within the polyA site of the selection cassette. **(C)** The null allele is reverted to a conditional allele by Fip-recombination. **(D)** After excision of the frameshifting exon by Cre-recombination a premature stop codon in exon 3 (E3-asterisk) leads to the activation of the non-sense mediated decay mechanism and degradation of the mRNA. X and Y depict hypothetical restriction endonucleases for Southern – hybridization with the 5’ and 3’ external probes (black horizontal bars). Arrows show primer positions for screening correct targeted events by long-range PCR (LR1, LR2, LR3, LR4), for confirming Fip-recombination (F1, F2) and Cre-recombination (C2).

*Color image of this figure appears in the color plate section at the end of the book.*

of the endogenous and the LacZ-neo fusion that will terminate in the polyA site of the cassette. This “KO-first” or “null” allele can be reverted to a conditional allele by excising the selection cassette with Fip-mediated recombination in the cells or in mice (Testa et al., 2004). Administration of Cre will delete the critical exon and result in a frame-shifted allele. This so-called “multi-purpose” allele strategy is adopted by the international knockout mouse consortia EUCOMM (European Conditional Mouse Mutagenesis) and KOMP (Knock Out Mouse Project).

Recombineering technology and the availability of an almost complete mouse genome sequence have facilitated enormously the generation of conditional targeting vectors (Zhang et al., 1998). Instead of screening genomic libraries by hybridization for identifying the genomic clone or using PCR for amplifying homology arms, one can start the generation of the targeting vectors using already existing genomic clones in form of BACs. The cloning exercises can be accomplished in a high-throughput manner allowing the parallel production of targeting constructs in very short time (Skarnes et al., 2011). This constantly increasing collection of targeted vectors is an open resource supported by the EU through the EUCOMM program ([www.eucomm.com](http://www.eucomm.com)). The resource also contains targeted mESC lines, which can be viewed in the International Knockout Mouse Consortium (IKMC) web portal ([www.knockoutmouse.org](http://www.knockoutmouse.org)) and are available upon request to the scientific community (Bradley et al., 2012). The depository also contains a resource of vectors for targeted deletion of micro-RNAs in mouse ES cells (Prosser et al., 2011).

## CHROMOSOME ENGINEERING

A number of human diseases and types of cancer are caused by chromosomal rearrangements such as deletions (Prader-Willi Syndrome), insertions (Acute Myeloid Leukaemia), duplications (Down syndrome), translocations (different types of Leukaemias) and inversions (Haemophilia type A) (van der Weyden and Bradley, 2006). Chromosomal rearrangements have been identified by cytogenetic studies and in the cases of translocations sequencing technology revealed the underlying DNA sequence enabling to map the exact position of the break point that occurred in the translocated chromosomal regions. Still the genes that are responsible for causing the phenotypic changes in cases where duplications or large-deletions have occurred remain unknown. Site-specific recombination strategies (Cre/loxP) enable the generation of disease models of human chromosomal rearrangements in ES cells and mice. For translocation models, chromosome engineering involves the insertion of a single loxP site together with a selection marker and the 5' half site of the *Hprt* minigene by gene targeting into a predefined position in the genome, for example into the mouse orthologue of the respective human gene. Subsequently a second loxP site is inserted again by gene targeting together with another selection marker and the 3' half site of the *Hprt* minigene in the chromosome where the mouse orthologue of the human translocation partner lies. Translocation between the two engineered chromosomes occurs upon Cre expression, which can be constitutive or inducible either in an ubiquitous or tissue-specific manner. The translocation event is selected by the reconstitution of the *Hprt* minigene, which confers resistance to HAT containing medium

(Smith et al., 1995). A collection of insertion type targeting vectors that contain end-sequenced genomic fragments and either the 5'-*Hprt* or 3'-*Hprt* minigene is publicly available under the name "Mutagenic Insertion and Chromosome Engineering Resource" (MICER) (Adams et al., 2004). For establishing an *in vitro* human disease model in mouse ES cells a prerequisite is that the synteny between mouse and human is conserved and that the orientation of the genes in the chromosome with reference to the centromere is the same between human and mouse. If this is not the case, then translocation will result into two products: a dicentric chromosome and an acentric chromosome that will get lost upon cell division. For large-scale deletions, first a single loxP is inserted by gene targeting in a defined position. The second loxP site can be inserted either by gene targeting in a certain position or randomly using retroviral vectors or other type of gene-trap vectors (Ramirez-Solis et al., 1995; Su et al., 2000). The advantage of the latter strategy is that it uses a single vector for generating a library of ES cells in which the position of the first loxP site is fixed and the position of the second varies (Su et al., 2000). Assuming that the integration of the second loxP site is mapped and is on the same chromosome as the one that was initially targeted, then one can make a series of deletions that have different lengths and so identify the gene that is causing the phenotype in the respective human chromosomal deletion disorder.

## RECOMBINATION MEDIATED CASSETTE EXCHANGE (RMCE)

This approach enables the exchange of a genomic fragment flanked by site-specific recombination target sites with another one. The conversion of one type of allele to another occurs in the already genetically engineered cells, for example a KO allele can be converted to a conditional allele, as has been shown for targeted deleted microRNA alleles (Prosser et al., 2011) or a reporter allele that is coding for GFP can be converted to a Cre-recombinase expressing allele. The piece of DNA to be exchanged can be targeted or randomly integrated in the genome. The exchange is more efficient when the fragment is flanked by an heterotypic and an homotypic site or with sites recognized by two different recombinases (Osterwalder et al., 2010).

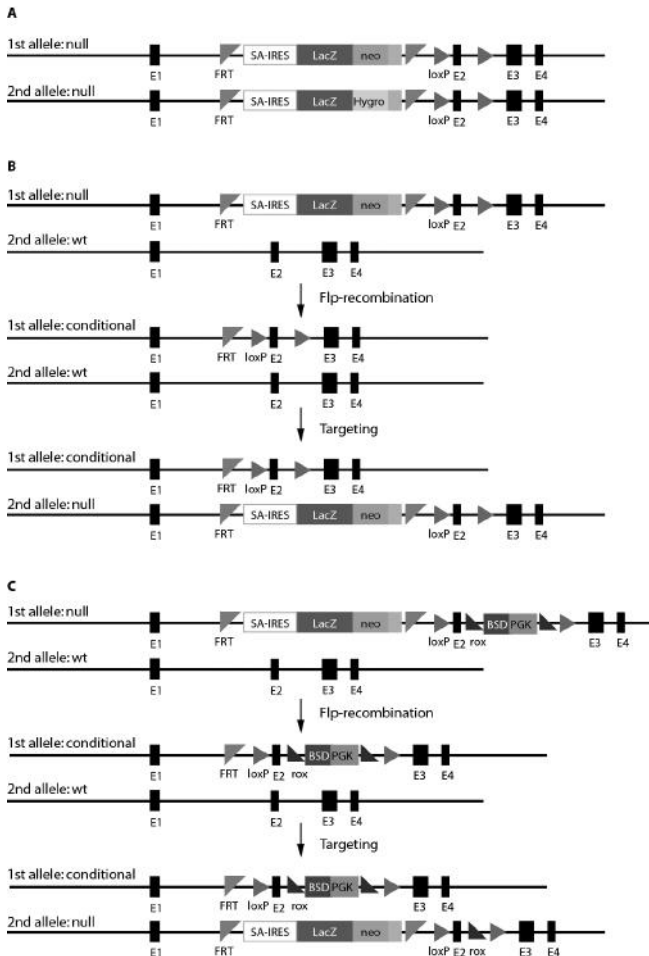
By exploiting the principle of RMCE, Wallace et al. developed a strategy termed Recombinase-mediated genomic replacement (RMGR) that allows the exchange of large genomic segments (>100 kb) in the mouse genome (Wallace et al., 2007). The technology involves first the modification of the mouse genome by inserting recombination target sites flanking the piece to be exchanged in mES cells and then the modification of a human BAC (or large construct) by adding recombination target sites and the appropriate selection markers. RMGR allows the development of human disease models in the mouse genome, such as  $\alpha$  thalassemia by replacing the mouse  $\alpha$  globin

locus with an orthologous human gene that lacked the  $\alpha$  globin regulatory domain (Wallace et al., 2007).

## TARGETING THE SECOND ALLELE

ES cells are diploid and in order to study loss of gene function it is necessary to target both alleles, or isolate ES cells from mice that carry the desired mutation. There are different methods for targeting both alleles, each of them with certain advantages and disadvantages:

- a) High selection pressure. In cells in which one allele is already targeted by inserting a selection marker such as the neomycin resistance gene, the second allele can be converted from wt to a mutated one by increasing the G418 concentration (Mortensen et al., 1992). This is a rare event that occurs with a rate of about  $10^{-5}$  per cell per generation and is due either to gene conversion or to a cross-over between sister chromatids during mitosis (mitotic recombination) or loss of the whole chromosome and duplication of the one that bears the targeted insertion. The exchange rate can be increased at least by an order of magnitude with the help of site-specific recombination (Cre) between chromosomes. This strategy requires the engineering of both alleles of a gene by inserting a loxP site using homologous recombination in a subcentromeric position. Then the neomycin resistance gene is targeted into a locus on the same chromosome and distal to the loxP sites. Biallelic mutant clones can be selected after expressing the Cre-recombinase and with high doses of G418 (Koike et al., 2002). This strategy requires pre-engineering of chromosomes with loxP sites, which is a laborious process. Another drawback of this method is that it can result in homozygosity of recessive genes or in homozygosity of imprinted loci.
- b) Sequential targeting. Once the first allele is targeted then the second allele can be targeted using a construct that contains a different selection marker. For example the first allele is targeted using a neomycin resistance gene and the second with a vector that contains the hygromycin resistance gene (Figure 3A). After electroporation of the second targeting construct the cells are selected using both antibiotics (G418 and Hygromycin B). There are cases in which this is not possible because the gene is essential in ES cells and the targeting cassette disrupts gene function. Targeting the first allele with a vector in which the selection cassette can be removed by site-specific recombination restores the function of the allele. In the targeting vectors that rely on the multi-purpose allele strategy (Testa et al., 2004), the selection



**Figure 3.** Targeting the second allele. **(A)** If the gene is not essential in ES cells, the second allele can be targeted with the same targeting construct after exchanging the selection marker (from neo to Hygro) using recombineering in bacteria. The cells are selected for resistance to G418 and Hygromycin. **(B)** If the gene is essential in ES cells, then the first targeted allele is reverted to a conditional allele by Flp-recombination. The second allele is targeted with the same targeting construct and a theoretical probability of 50%. **(C)** If the gene is essential in ES cells, the first allele can be targeted by a construct that contains in addition to the SA-IRES-LacZ-neo-pA cassette, a PGK-BSD (blasticidin) cassette positioned downstream of the critical exon and flanked by rox sites (blue triangles) for Dre-recombination. The first targeted allele can be converted to a conditional after Flp-recombination. This configuration resembles the wt allele, assuming that the PGK-BSD cassette, which is inserted in an intron, does not interfere with the expression of the gene. The second allele is targeted with the same targeting construct after removing the PGK-BSD cassette by Dre-recombination in bacteria. The cells are selected for resistance to G418 and blasticidin.

*Color image of this figure appears in the color plate section at the end of the book.*



cassette contains the LacZ reporter and the neomycin resistance gene and is flanked by FRT sites. Complete excision of the cassette after Flp-recombination is confirmed by sensitivity to G418 and loss of beta-galactosidase expression (Tate and Skarnes, 2011). Then the other allele can be targeted with a theoretical probability of 50% using the same vector. This is not always the case and there is a tendency of retargeting the already targeted allele resulting in lower efficiency than the expected for targeting the other allele (Figure 3B). Targeting vectors that have an additional selection marker downstream of the floxed exon can be used to improve efficiencies for double targeting. The first allele is targeted with a vector that contains two selection cassettes: the LacZ-neo cassette flanked by FRT sites upstream of the floxed critical exon and a PGK-BSD (blasticidin) cassette flanked by rox sites for Dre recombination and positioned downstream of the floxed exon (Figure 3C). This vector can be modified to contain only the LacZ-neo or the PGK-BSD cassette by Flp- or Dre-recombination in bacteria. After successful targeting and removal of the LacZ-neo cassette by Flp-recombination, the second allele can be targeted using the vector that contains only the LacZ-neo cassette. With this strategy, double selection can be applied: Blasticidin to maintain the first targeted allele and G418 to target the second allele (Figure 3C).

- c) Double targeting with engineered nucleases. It has been reported that gene targeting using ZFNs or TALENs can result in both alleles being modified (Hockemeyer et al., 2011). This is due to the high efficiency of gene targeting after introducing a double strand break by the engineered nucleases. The advantage is that this strategy requires only a single targeting vector that will repair the DSB by homology driven repair. But again if the gene is essential in ES cells, then double targeting will not be possible such as in the case of targeting the hOCT4 locus in hESC with a C-terminal GFP tag using TALENs (Hockemeyer et al., 2011).
- d) Targeting in Bloom syndrome ES cells. The gene mutated in Bloom syndrome is a tumor suppressor gene encoding an homologue of the *E. coli* RecQ DNA helicase and Bloom deficient ES cells show an increased frequency of mitotic recombination as compared to wt cells. Bloom deficient ES cells are generated either by targeted disruption of both Bloom helicase alleles or by tetracycline regulated inactivation of the helicase expression (Luo et al., 2000; Yusa et al., 2004). The latter is a more controlled way to inactivate the helicase and avoid the constant accumulation of other mutations in a homozygous state during prolonged culture, which may interfere with the interpretation of the phenotypic analysis. Getting homozygous alleles from a targeted heterozygous or hemizygous event can occur with a frequency of

$2-4 \times 10^{-4}$  per cell per locus and generation (Luo et al., 2000; Yusa et al., 2004). This frequency is at least an order of magnitude higher than the one observed in wt cells in an experiment of inducing homozygosity of a targeted *Fasl* (Fas ligand) allele by selecting in high doses of G418 (Yusa et al., 2004).

## LARGE-SCALE MUTAGENESIS

There are two main methods for genome wide mutagenesis in ES cells. The one is phenotypic driven and relies on chemicals (e.g., ENU mutagenesis), and the other is insertional mutagenesis using DNA based vectors.

### CHEMICAL MUTAGENESIS (ENU)

N-ethyl-N-nitrosourea (ENU) is an alkylating agent that causes base substitutions in DNA with a preference to AT base-pairs (Justice et al., 1999). Mutagenesis screens in mice after ENU injection in male germ cells and subsequent breeding to homozygosity has revealed a number of interesting mutations related to phenotypes (Gondo et al., 2010). ENU mutagenesis in ES cells showed a mutation rate of 1 in 1000 at the dose of 0,35 mg/ml, which is similar to the one observed in mouse germ cells (Chen et al., 2000). Moreover ES cells maintain their germline competence even after exposure to a high dose of ENU (0,35 mg/ml to 0,5 mg/ml) (Chen et al., 2000). An ENU screen in ES cells for resistance to oxidative stress induced by the drug Paraquat revealed clones that produced mouse lines from which isolated skin derived fibroblasts showed also resistance to oxidative stress (Chick et al., 2009).

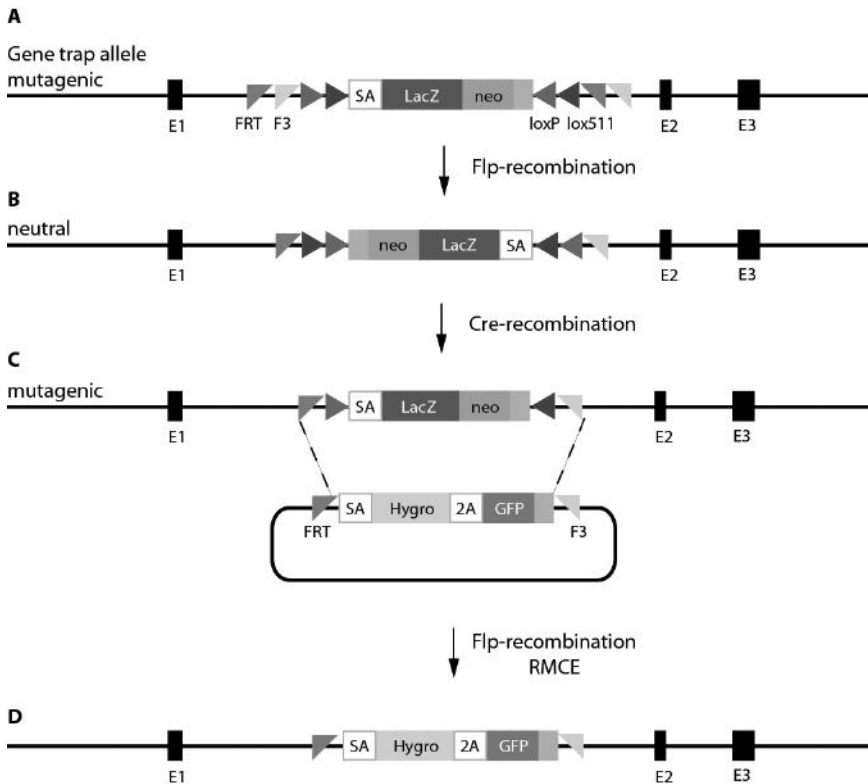
The advent of Bloom's deficient ES cells in which loss of heterozygosity can occur with a high frequency and lately the derivation of haploid ES cells from parthenogenetic oocytes provide a better platform for ENU mutagenesis screens. So far ENU mutagenesis of Bloom's deficient ES cells combined with a screen for aerolysin resistance identified homozygous mutants in half of the genes involved in the glycosylphosphatidylinositol (GPI)-anchor biosynthesis (Yusa et al., 2004). There are at least 23 genes involved in the GPI-anchor pathway and mutations in each one of them causes resistance to the drug aerolysin, which kills cells with GPI-anchors. The disadvantage of ENU mutagenesis is the difficulty to identify the single base-pair substitution that has caused the observed phenotype. This can be done by whole genome sequencing or sequencing on genomic arrays, methods that are still expensive for applying them on a routine basis.

## GENE TRAPPING

Gene trapping is the introduction of DNA-based vectors that integrate randomly across the genome causing insertional mutations. Trap vectors are designed to hijack the expression of an endogenous gene that is close to the insertion site. There are different types of trapping vectors: a) enhancer traps, b) promoter traps and c) gene traps (Stanford et al., 2001). Enhancer trap vectors contain a weak promoter and so they can trap enhancer elements near the site of integration in the genome. Promoter trap vectors lack promoter elements and usually integrate into transcribed exons. At last, gene trap vectors depending on their design can capture the expression of transcriptionally active genes or integrate into transcriptionally silent genes that will be activated during development. Usually, gene trap vectors contain a strong splice acceptor followed by a selection marker/reporter gene and poly-adenylation sequences (Hansen et al., 2003). A fusion transcript is generated from the upstream sequences of the endogenous gene and the selection marker/reporter gene of the inserted vector. The captured endogenous transcript can be identified by 5' RACE (rapid amplification of cDNA ends) and the exact site of integration can be mapped using inverse or Splinkerette PCR (Horn et al., 2007).

The frequency of gene trapping using promoterless gene trap vectors is relative to the expression level of the gene. To mutate genes that are not expressed in ES cells, poly-A-trap vectors are used, which contain a promoter that drives a selection marker and a splice donor but lacks a poly-adenylation signal (Niwa et al., 1993). The expectation is that the transcript of the selection marker will be terminated using a downstream endogenous poly-A signal. Indeed, poly-A-trap vectors integrate into transcriptionally silent genes but show a preference for insertion into the last intron. This phenomenon is explained by the fact that if the vector is integrated into introns that are upstream, then the premature stop codon of the selection marker can activate the non-sense mediated decay (NMD) mechanism of mRNA degradation (Shigeoka et al., 2005). To avoid integration into the last intron, Shigeoka et al, included an IRES element in their vectors positioned between the selection marker and the splice donor. Indeed this so called UPATrap vectors were shown to integrate in upstream introns of non-expressed genes (Shigeoka et al., 2005).

The challenge is to mutate every single gene in the genome and this has not been achieved yet. Gene trap vectors relying on the SA principle of trapping can trap mainly genes that are expressed in ES cells. Moreover there are different vector types and some of them show preferential insertion into hot spots. Retroviral vectors integrate preferentially into the 5' half of the genes whereas DNA vectors or transposons do not show a preference (Hansen et al., 2003). Therefore in order to achieve a genome wide saturated



**Figure 4.** Conditional gene trapping based on the Flip-Excision (FIEEx) strategy. **(A)** A gene trap cassette consisting of a splice acceptor (SA)-LacZ-neo fusion and a polyA (grey box) is flanked by a series of site-specific recombination target sites in inverted orientations (FRT, F3, loxP, lox511). The transcript of the gene is captured by the SA and is terminated within the pA site. **(B)** After Flp-recombination and the first inversion, the two F3 sites come into the same orientation and the FRT site that is positioned in-between is deleted. A second inversion by Flp-recombination is not possible because two incompatible sites (F3 and FRT) are flanking now the cassette. In this orientation the cassette cannot capture the transcript of the gene and is neutral. **(C)** The cassette can be inverted once again to the mutagenic orientation by Cre-recombination. **(D)** Recombination mediated cassette exchange (RMCE) can be used to convert the LacZ-neo containing gene-trapped allele to another reporter. In this example Flp mediates recombination between two heterotypic sites and exchanges the trapped cassette to one containing the Hygromycin resistance gene (for selection) and GFP separated by 2A peptide.

*Color image of this figure appears in the color plate section at the end of the book.*

insertional mutagenesis a combination of gene trap vectors should be used. Still trapping single exon genes is going to be difficult. So far there is a single incidence reported that the U3 betageo vector can disrupt an intronless gene

(Hansen et al., 2003). Gene trapped mouse ESC lines are publicly available from the International Gene Trap Consortium ([www.genetrap.org](http://www.genetrap.org)).

## CONDITIONAL GENE TRAPPING

In their original design, gene trap vectors did not have the option for conditionality. A strategy that relies on directional site-specific recombination was employed for generating conditional gene traps (Schnutgen et al., 2005). In this strategy, termed flip-excision (FLEX) the mutagenic cassette is flanked by four heterotypic sites from each side (Frt, F3 for Flp- and loxP, lox511 for Cre-recombination). The four upstream sites are in the opposite orientation to the downstream sites. Upon Flp-recombination the mutagenic cassette will be inverted and bring the upstream Frt, F3 sites close to the downstream Frt and in the same orientation. This will result into the deletion of the fragment between the two Frt (homotypic) sites, resulting in a product that has an F3 site upstream and an Frt site downstream. Flp-inversion renders the cassette non-mutagenic because the poly-A signal is now in the anti-sense orientation (unless there is an anti-sense transcript). The mutation can be reinduced by Cre-recombination, which will result in a second inversion of the cassette and restoration of its mutagenic function (Schnutgen et al., 2005).

Another advantage of available ES libraries containing conditional gene traps is their versatility. The gene trap vectors contain site-specific recombination target sites that can be used as a template for RMCE. From this already existing resource new alleles can be generated by transfecting into the cells constructs that carry the compatible target sites and the new gene of interest such as fluorescent proteins, affinity purification tags for proteomics, recombinases, etc. (Schnutgen et al., 2011; Singla et al., 2010). A large proportion of gene trap vectors integrate into the first intron of the gene, so using RMCE and an exchange vector that contains a splice acceptor, a protein tag (nLAP) and a splice donor, is like introducing an additional exon into the 5' region of the gene (Poser et al., 2008; Schnutgen et al., 2011). This enables the N-terminal tagging of the trapped gene and downstream proteomic analysis.

## GENETIC SCREENS IN BLOOM'S DEFICIENT ES CELLS

Bloom's deficient ES cells offer new possibilities for contacting phenotypic driven genetic screens. Functional screens using either retroviral gene trap vectors or piggyBac mediated transposition in Bloom's deficient ES cells identified mutations in known but also novel genes involved in the DNA mismatch repair pathway (Guo et al., 2004; Wang et al., 2009). Those screens

were performed in the same lab and interestingly the screen using the retroviral gene trap vector didn't reveal the mismatch repair genes *Msh2*, *Pms2* and *Mlh1* but most of the insertions landed in *Msh6*. In contrast the screen using the piggyBac vector identified mutations in *Msh2*, *Pms2* and *Mlh1* genes. This underlines the neutrality of piggyBac vectors regarding integration site preference as compared to retroviral vectors (less hot spots than retroviral insertions). Using also a piggyBac transposon Guo et al. screened for genes involved in regulating pluripotency in ES cells and identified multiple hits in the *Tcf3* gene (Guo et al., 2011). Phenotype driven genetic screens are not trivial and the successful outcome depends largely on the way the read out is set up (how sensitive and specific is the selection). Although the rate of mitotic recombination in Bloom deficient ES cells is considered to be high (at a range of  $10^{-4}$ ), if there is no way to select for the loss of heterozygosity, then this event will be masked by the other heterozygous cells. Therefore gene trap cassettes that allow for selection of the loss of heterozygosity of the mutated allele have been developed. These cassettes contain two selection markers of which only one is expressed in the heterozygous state. The expression of the other selection marker is turned on using site-specific recombination (Cre-expression). Loss of heterozygosity can thus be selected because in the cells that express both selection markers and are resistant to both antibiotics has occurred a sister chromatid exchange (Horie et al., 2011; Huang et al., 2012). Nevertheless, cells that have acquired aneuploidy or trisomy of the mutated chromosome also occur, meaning that the clones should be carefully analysed before contacting any phenotypic screens (Huang et al., 2012). More important, rescue of the phenotype by overexpressing the wt-copy of the mutated gene or by removal of the gene trap cassette using site-specific recombination is necessary for proving that the observed phenotype is caused by the mutated gene.

## GENETIC SCREENS IN HAPLOID ES CELLS

The term haploid refers to a single set of chromosomes and comes from the Greek word haplos, which in modern Greek also means simple. However haploidization of the mammalian genome is not a simple but rather a complicated process that occurs during meiosis. A mammalian oocyte completes meiosis upon fertilization giving rise to the zygote, which will undergo a certain number of cell divisions before starting differentiating. An unfertilized oocyte can also be triggered to undergo cleavage using chemicals ( $\text{SrCl}_2$  or Ethanol), a phenomenon called parthenogenesis. Parthenogenetic embryos can reach the blastocyst stage of development but cannot develop to term because of abnormal dosage of gene expression attributed to parental imprinting. Human and mouse ES cells can be isolated from parthenogenetic blastocysts and carry a diploid set of chromosomes.

However a small proportion of the parthenogenetic ES cells carry an haploid set of chromosomes and can be sorted by fluorescence cytometry (Elling et al., 2011; Leeb and Wutz, 2011). Those ES cells are not stably maintained haploid and tend to become diploid, so one has to sort the cells after every passage for keeping the single set of chromosomes. Nevertheless, haploid ES cells express pluripotency markers, can be differentiated into all three germ layers and generate chimaeras after injection into the blastocyst (differentiated cells undergo diploidization). More important haploid ES cells can be used for forward and reverse genetic screens. Elling et al. transduced the cells with a reversible gene trap vector and performed a screen for resistance to the bioweapon ricin. This forward genetic approach revealed some already known targets but also a new gene (*Gpr107*) involved in mediating ricin toxicity (Elling et al., 2011). Also a screen for mismatch repair using a piggyBac transposon vector revealed *Msh2*, a gene known to be involved in this pathway (Leeb and Wutz, 2011; Yang et al., 2012). Haploid ES cells can be also targeted with the same efficiency as diploid ES cells. Even if the cells acquire diploidy with prolonged passage or after differentiation, they are still valuable because they will also duplicate the chromosome that carries the mutation. The question is whether haploid cells can contribute to the germline. Androgenetic haploid ES cells were also generated by a more elaborate approach, which involved first the enucleation of the female genetic material in the oocyte and then intracytoplasmic injection of a single sperm followed by chemical activation to undergo cleavage (Yang et al., 2012). Also it is possible to derive haploid cells by fertilization and then enucleation of the female pronucleus. These cells show pluripotent characteristics and are amenable to gene targeting but also tend to undergo diploidization upon differentiation as already observed with their parthenogenetic counterparts. Androgenetic haploid ES cells contribute to chimaeras after injection into the blastocyst but they also contribute to the germline after injection into MII (Metaphase II) oocytes followed again by chemical activation to pursue cleavage (Yang et al., 2012). Despite the technical difficulties for obtaining and maintaining haploid ES cells, the fact that the diploid hurdle can be overcome *in vitro* makes them a very useful model for conducting genetic screens.

## CONCLUSIONS

Recombineering technology facilitated the generation of targeting vectors and the engineering of large constructs. Today there are publicly available resources that contain ready to go targeting vectors or genetically engineered mouse ESC lines. The initial difficulties encountered with the genetic manipulation of human pluripotent stem cells can be overcome with the help of engineered nucleases (TALENs and ZFNs). Still publicly available

resources for human cells that encompass targeting constructs or engineered cell lines, as is the case for mESC are not yet developed. The mouse system remains the protagonist in our efforts to understand gene function by manipulating its genome. Moreover, the recent derivation of mouse haploid cells enables the fast performance of both forward and reverse genetic screens. It is a matter of time, until the developed technologies can be applied to other mammalian genomes and deliver information, which will help us understand gene function. The next challenge is in front of us: combinatorial mutagenesis for deciphering genetic interactions.

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## **ABBREVIATIONS**

|        |                                                             |
|--------|-------------------------------------------------------------|
| ESC    | Embryonic stem cells                                        |
| RNAi   | RNA interference                                            |
| BACs   | bacterial artificial chromosomes                            |
| AV     | Adenovirus                                                  |
| HSV    | Herpes Simplex Virus                                        |
| IRES   | internal ribosomal entry site                               |
| ITRs   | inverted terminal repeats                                   |
| SB     | Sleeping Beauty, transposase                                |
| S/MARs | scaffold/matrix-attachment regions                          |
| UCOE   | ubiquitous chromatin opening element                        |
| HSV TK | Herpes Simplex Virus Thymidine Kinase                       |
| FIAU   | 1-(-2-deoxy-2-fluoro-1-b-D-arabinol-furanosyl)-5-iodouracil |
| DTA    | Diphtheria Toxin A                                          |
| PGK1   | phosphoglycerate kinase1                                    |
| AAV    | Adeno-associated virus                                      |
| HDAdVs | Helper Dependent Adenovirus                                 |
| NHEJ   | Non Homologous End Joining                                  |
| ZFNs   | Zinc Finger Nucleases                                       |
| ZFP    | zinc finger protein                                         |
| TALENs | Transcription Activator Like Effector Nucleases             |
| RVDs   | repeat variable diresidues                                  |
| EUCOMM | European Conditional Mouse Mutagenesis                      |
| KOMP   | Knock Out Mouse Project                                     |
| IKMC   | International Knockout Mouse Consortium                     |



|      |                                                         |
|------|---------------------------------------------------------|
| RMCE | Recombination Mediated Cassette Exchange                |
| RMGR | Recombinase-mediated genomic replacement                |
| ENU  | N-ethyl-N-nitrosourea, used for chemical mutagenesis    |
| NMD  | non-sense mediated decay, mechanism of mRNA degradation |

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# About the Authors

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## Chapter 1



**Stephen Dalton** is Professor and GRA Chair in Molecular Cell Biology in the Department of Biochemistry and Molecular Biology at the University of Georgia. Dr. Dalton received a BSc (Hons.) from Flinders University and his Ph.D. from the University of Adelaide, Australia. This was followed by a post-doctoral position at the Imperial Cancer Research Fund (ICRF; Cancer UK), London and faculty appointments at the Roche Institute for Molecular Biology in New Jersey and the University of Adelaide.



**Amar Singh** earned his PhD in Molecular Cell Biology in the laboratory of Dr. Naohiro Terada, at the University of Florida in 2006. Amar is currently a postdoctoral associate in Stephen Dalton's lab at the University of Georgia. His research interests include stem cell pluripotency, cell cycle regulation, cell signaling and mechanisms of cell fate commitment. Amar has published articles on the topic of pluripotent stem cells top-tier journals including *Cell Stem Cell*, *Stem Cells*, *Circulation* and has authored several reviews with Dr. Dalton for the *Faculty of 1000*.

## Chapter 2



**Paula Alexandre** is a Principal Investigator and Lecturer at Institute of Child Health, University College London. She studies the molecular mechanisms that regulate neurogenesis during brain development. Her lab currently uses live-imaging and genetic approaches in zebrafish embryos to determine the molecular mechanisms that regulate self-renewal and differentiation in stem cell niches and developing neural tube.





**Yoichi Kosodo** is a Principal Investigator and Associate Professor of Department of Anatomy at Kawasaki Medical School. His lab investigates mammalian neurogenesis by pursuing a concept how tissue and cells reciprocally influence and regulate each other to form a robust architecture of our brain. To illustrate such regulatory systems of neurogenesis, the group has been constructing novel models, and examining their consistency by applying interdependent experimental approaches, such as methods of molecular cell biology, live-imaging, quantitative biology, and biomaterials.

## Chapter 3



**Frank Rosenbauer** is a graduate in biology of the University of Würzburg, Germany, and received his doctoral degree from the Free University of Berlin, Germany. After postdoctoral training with Daniel Tenen at Harvard University, USA, he became an independent group leader at the Max Delbrück Center for Molecular Medicine in Berlin in 2005. In 2011, he became full professor and director at the Institute for Molecular Tumor Biology of the University of Münster, Germany. His laboratory is mainly interested in understanding how epigenetic and transcriptional networks control early hematopoiesis and cancer development.



**Margaret ("Peggy") Goodell** holds the Vivian L. Smith Chair in Regenerative Medicine at Baylor College of Medicine where she has been on the faculty since 1997 and directs Stem Cells and Regenerative Medicine Center. Goodell received her PhD from Cambridge University and underwent post-doctoral training at MIT where she developed the "side population" (SP) method for hematopoietic stem cell purification based on their efflux of the vital dye Hoechst 33342. Her current research is focused on the fundamental mechanisms that regulate hematopoietic stem cells, and how those regulatory mechanisms go awry in blood cancers. She has received the Stohlman Scholar Award from the Leukemia and Lymphoma Society, and the Edith and Peter O'Donnell Award in Medicine, and the Damashek Prize from the American Society of Hematology. Goodell has served on the editorial boards of *Blood*, *Cell Stem Cell*, and *PLoS Biology*. Goodell directs a laboratory of about 20 trainees.

## Chapter 4



**June-Won Cheong** received MD in 1994 and PhD in 2006 from Yonsei University College of Medicine, Seoul, Korea. He is now an associate professor of Hematology at Yonsei University and director of Blood Cancer Clinic, Severance Hospital, Seoul, Korea. During he had worked with Prof. Toshio Suda at Keio University as a visiting assistant professor, his research interest was the megakaryopoiesis and stem cell biology.



**Ayako Nakamura-Ishizu** is a post-doctorate researcher in Dr. Toshio Suda's lab in Keio University. She is an M.D. from Tokyo Women's Medical University. After training in clinical hematology, she returned to academics and received a Ph.D. degree in medical science, specializing in anatomy and histochemistry. Her research interest lies in the study of the hematopoietic stem cell niche.



**Toshio Suda** is an MD from Yokohama City University School of Medicine. He was Professor of the Department of Cell Differentiation at Kumamoto University School of Medicine, now he is Professor of Developmental Biology at Keio University. Prof. Makio Ogawa and Toshio Suda at Medical University of South Carolina, proposed a stochastic model in the differentiation of stem cells. This is evaluated as a classical work showing the independent differentiation of stem cells from the growth factors. Toshio Suda identified the niche for hematopoietic stem cells and subsequently established the new field of oxidative stress and stem cell aging. The interaction of stem cells and niche is one of hot topics for the stem cell biology.

## Chapter 5



**Vaibhav P. Pai** is Research Associate in Dr. Michael Levin's laboratory at Tufts University's Center for Regenerative and Developmental Biology. He received his bachelor's degree in microbiology and is trained as a biophysicist (Masters). He began his research as a doctoral student in Dr. Nelson D. Horseman's laboratory in the Systems Biology and Physiology program at University of Cincinnati, USA where he studied the biophysical

homeostatic mechanisms governing the regenerative capabilities of mammary epithelium. He started his postdoctoral research in Dr. Michael Levin's laboratory at Tufts University focusing on studying endogenous bioelectric signals as patterning information conduits during embryonic organ system formation particularly neural systems (eye and brain). This knowledge allows application of bioelectric information processing modality in regenerating organ systems for treatment of birth defects, cancers and traumatic injuries. This effort combined with Dr. Levin's laboratory expertise in Developmental and Regenerative Biology, Computational and Synthetic Biology and Mathematical Modeling provide a rich and exciting environment to push boundaries of our knowledge and have significant impact on our understanding of information encoding and processing in biological systems. <http://sites.tufts.edu/vaibhavpai/>.



**Michael Levin** was originally trained in computer science, working in artificial intelligence and information processing. His interest in the engineering of systems with adaptive behavior and self-repairing abilities led him to study the robust patterning mechanisms of living systems. After a PhD with Cliff Tabin at the Genetics department of Harvard Medical School, and a post-doc with Marc Mercola at the Cell Biology department at

HMS, he started his own lab at Forsyth Institute. The Levin lab studies storage, processing, and communication of patterning information among living cells during regeneration, development, and cancer suppression. Our specific focus is on endogenous bioelectric cues—standing voltage gradients in complex tissues that serve as prepatterns for anatomical polarity, organ identity, and gene expression. Our development of state-of-the-art tools for molecular-level tracking and manipulation of bioelectric signals has allowed the discovery and manipulation of ionic controls of eye induction, tail regeneration, left-right asymmetry, regenerative polarity control, and stem cell regulation. Our computational modeling efforts are revealing how real-time dynamics of physiological networks guides establishment, repair, and maintenance of morphology. The group moved to Tufts University in 2009, to be closer to interdisciplinary collaborators in computer science, bioengineering, and cognitive science. [www.drmichaellevin.org](http://www.drmichaellevin.org).

## Chapter 6



**Kenneth R. Boheler** received his Ph.D. in Physiology and Pharmacology from the University of California San Diego and received a National Science Foundation post-doctoral fellowship to train in Cardiac Molecular Biology at INSERM U127 in Paris France. He subsequently took an academic position in the Department of Cardiothoracic Surgery at the Imperial College School of Medicine in London to study the

function and role of calcium handling proteins in development and disease. It was during this period when he established long-term collaborations with the Developmental Biologist Antoon Moorman (Amsterdam, Netherlands) and the Stem Cell Biologist Anna Wobus (Gatersleben, Germany). These interactions ultimately set the stage for his long-term interest in cardiac developmental biology and his focus on embryonic stem cells differentiation to cardiomyocytes. After moving to the National Institute on Aging, Dr. Boheler and his group were the first to show the critical role of the cardiac ryanodine receptor in setting the beating rate of cardiomyocytes, were the first to demonstrate that the distal upstream region of the Na-Ca exchanger promoter was cardiac restricted and expressed in the cardiogenic plate, and they led efforts to unravel the mechanisms responsible for cardiomyocyte differentiation *in vitro* through comprehensive transcriptomic, cellular and functional assessments. His laboratory is currently transitioning to Hong Kong, where he will continue to work on biological mechanisms underlying embryonic stem cells (ESCs) pluripotency; cardiomyocyte differentiation; cardiomyocyte cell cycle progression, apoptosis and maturation; as well as a multifaceted program designed to evaluate the suitability of human derived induced pluripotent stem cells (iPSCs) generated from patients with cardiovascular diseases for basic research, disease modeling and potential therapeutic applications.

## Chapter 7



**Rachel Golub** is a PhD and an assistant professor at the university Denis Diderot-Paris 7. Her group contributes to the characterization of early hematopoiesis in the fetal spleen in the Lymphopoiesis Unit headed by Ana Cumano at Pasteur Institute. Her research interests combine the development of both the hematopoietic progenitors and the stromal populations that sustain peripheral hematopoiesis.

Embryonic peripheral lymphoid organs such as the spleen and lymph nodes are early colonized by innate lymphoid cells. Then, innate lymphoid cell development and regulation is under consideration from the fetal liver precursor to the peripheral mature stage.

E-mail: [rachel.golub@pasteur.fr](mailto:rachel.golub@pasteur.fr)



**Paulo Vieira** obtained his M.D. from the New University of Lisbon, Portugal. During postdoctoral work with Klaus Rajewsky at the Institute for Genetics in Cologne, he studied the humoral immune response in mice. He then joined the DNAX Research Institute in Palo Alto, California, where, as a post-doc in Kevin Moore's Lab, he cloned Interleukin 10. From 1995 to 2000, he set up and headed the Gene Targeting Facility at the

Gulbenkian Institute in Portugal.

In 2001, Paulo Vieira became an investigator at the Pasteur Institute in Paris, France where the main research focus of his group is on lymphocyte commitment and differentiation, with particular emphasis on B-cell development. Over the last few years, he studied the differences between lymphocyte development in fetal liver and adult bone marrow, unraveling the role of the cytokine TSLP in fetal B cell development as well as the essential role of IL-7 in adult B cell commitment.



**Ana Cumano**, MD, Lymphopoiesis Unit; Pasteur Institute; E-mail: [ana.cumano@pasteur.fr](mailto:ana.cumano@pasteur.fr)

In the last years our laboratory has been interested in stem cell biology and the development of the immune system. We contributed to the characterization of subsets of hematopoietic progenitors in fetal liver and to the description of the role of hematopoietic cytokines in lineage commitment. We identified newly

generated hematopoietic stem cells, in the first hematogenic compartment, the dorsal aorta.

2000-Member of EMBO

2009-Member of Scientific Council of the Pasteur Institute

2009-Member of the EMBO Courses and Workshops Committee.

## Chapter 8



**Iva Kelava** studied ecology at the University of Zagreb, Croatia, and then decided to pursue a PhD in the lab of Wieland Huttner, working on neural progenitors in mammalian neurogenesis. Her PhD work concentrated on comparative neocortical neurogenesis, with special focus on the differences and similarities between neural progenitor make up during development of lissencephalic and gyrencephalic mammalian species. Her general interest is evo-devo, and especially the role of the environment in shaping the development in individual species.

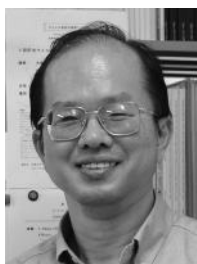


**Wieland B. Huttner** was born on 15 February 1950 in Hannover, Germany. He studied medicine at the Universities of Hamburg, Germany and Oxford, UK (1969–1975). In parallel to his studies, he carried out his doctoral thesis in physiological chemistry at the University of Hamburg (1972–1976). He then worked as post-doctoral fellow at the Max Planck Institute (MPI) of Experimental Medicine in Göttingen, Germany (1976–1977) and at Yale University, New

Haven, USA in the lab of the future Nobel Laureate Paul Greengard (1977–1980). Returning to Germany, he was recruited as Independent Junior Group Leader at the MPI for Psychiatry in Munich, Germany (1981–1985), and subsequently became Group Leader at the European Molecular Biology Laboratory (EMBL), Cell Biology Programme, in Heidelberg, Germany (1985–1990), where he also was responsible for the EMBL PhD Programme as Dean of Graduate Studies. He then moved to the University of Heidelberg as Professor of Neurobiology and Chair of the Institute of Neurobiology (1991–2000). In 1998, he became one of the Founding Directors of the MPI of Molecular Cell Biology and Genetics (MPI-CBG) in Dresden, Germany, where he has been working since 2001. Since then, he is also the Speaker of the International Max Planck Research School for *Cell, Developmental and Systems Biology* (IMPRS-CellDevoSys) in Dresden, and Honorary Professor of Neurobiology at the Technische Universität Dresden. Wieland Huttner won the Karl-Winnacker-Award (1985) and was awarded the Berthold Medal of the German Society for Endocrinology (2003). He has been engaged

in numerous additional academic functions, notably being appointed as Member of the German Council of Science and Humanities (2004–2010) and elected as Chair of the Scientific Council of the Max Planck Society (2009–2012).

## Chapter 9



**Ryoichiro Kageyama** received MD in 1982 and PhD in 1986 from Kyoto University. After postdoctoral training in National Cancer Institute, USA, I became Assistant Professor in 1989 and Associate Professor in 1991 at Kyoto University. There, I started the project of bHLH genes such as *Hes1* and *Math1* and analyzed their roles in neural development. I then moved to Institute for Virus Research, Kyoto University as Full Professor in 1997 and continued to study the functions of bHLH genes

in neural development. My current research involves studies on dynamics of gene expression during cellular proliferation and differentiation. We are analyzing the dynamic expression of *Hes1*, *Hes5* and their target genes in neural development by using the time-lapse imaging system. We are also interested in the roles of bHLH genes in adult neural stem cells and characterizing the significance of adult neurogenesis in brain functions.



**Toshiyuki Ohtsuka** received MD from Kyoto University in 1990, and after working as a neurosurgeon he attained PhD from Kyoto University in 1998. Then, he started his research on the Notch-Hes signaling in neural development with Dr. Ryoichiro Kageyama. From 2001 till 2003 he worked as a postdoc at Stanford University. He became Assistant Professor and is currently Associate Professor in Dr. Kageyama's laboratory at the Institute for Virus Research, Kyoto

University. He has been studying on the molecular mechanism of maintenance and differentiation of neural stem cells and mammalian brain development.



**Itaru Imayoshi** received Ph.D. in Bio studies from Kyoto University, Japan, in 2008. He has been studying on the cell-fate determination process of neural stem cells in Ryoichiro Kageyama's laboratory at Kyoto University. He is also working on the functional significance of adult neurogenesis in the mammalian brain.



**Hiromi Shimojo** received Ph.D. from Kyoto University, Japan, in 2008. She has been studying the functions of the Notch-Hes pathway in neural stem cell regulation in Ryoichiro Kageyama's laboratory at Kyoto University.

## Chapter 10



**Thomas A. Reh**, Ph.D. received his B.S. in Biochemistry from the University of Illinois, Champaign-Urbana in 1977 and his Ph.D. in Neuroscience from the University of Wisconsin-Madison in 1981. He went on to postdoctoral studies at Princeton and joined the Faculty of Medicine at the University of Calgary in 1984. He then moved to the Department of Biological Structure at the University of Washington-Seattle, as an Associate

Professor and was promoted to Professor in 1994. He served as Director of the Neurobiology and Behavior Program from 2001 to 2007. He has worked at the interface between development and regeneration, focusing on the retina, with the goal of applying the principles learned from developmental biology to design rationale strategies for promoting regeneration in the adult mammalian retina. He has published over 130 journal articles, reviews and books in the field of retinal regeneration and development.

## Chapter 11



**Martin Post** received his PhD from the University of Utrecht in The Netherlands in 1982. Following postdoctoral research training at Harvard Medical School, he was appointed as an Assistant Professor at Harvard in 1985. This was followed by a move to SickKids in 1986, where he is the Head and Senior Scientist of the Physiology & Experimental Medicine Program. He is a Professor at the University of Toronto

and is internationally recognized for his research on pulmonary development, injury and repair. He has authored more than 260 scientific papers, reviews and book chapters and holds a Canada Research Chair in Fetal, Neonatal and Maternal Health.



## Chapter 12



**Abigail Tucker** is a Reader in the Department of Craniofacial Development and Stem cell biology and the Department of Orthodontics at King's College London. She attained her PhD from Oxford University in the lab of Prof Jonathan Slack, before moving to London to work with Prof Paul Sharpe and Prof Andrew Lumsden. She started her own lab in 1999 at Guy's Hospital, moving to her current departments in 2002. Her lab is involved in

studying the formation of the face and patterning of the facial organs, such as the teeth, cranial glands, ear and jaw. She has over 60 publications in the field of craniofacial development and has worked with a number of animal models, including mouse, chick, *Xenopus*, zebrafish, snake and lizard, investigating how pattern is generated and the mechanisms behind evolutionary change.



**Professor Paul Sharpe** is the Dickinson Professor of Craniofacial Biology at Kings College London. He graduated with a degree in biology from York University (1977) and a PhD in biochemistry from Sheffield University (1981). Following postdocs in Sheffield, Wisconsin and Cambridge he became lecturer in molecular embryology at the University of Manchester in 1987 where he established a research group working

on the molecular control of tooth development. Following promotion to Reader in 1991 he was recruited to his present Chair at the Dental Institute of Guy's Hospital (later to merge with Kings College), where he established a new basic research department, the Department of Craniofacial Development. The department, of which he remains head, now consists of 13 academic research groups with over 80 research staff. From 2002–2008 he was Director of Research for the Dental Institute.

He has published over 250 research papers including articles in *Nature*, *Science*, *PNAS* and *Cell* press. He has supervised over 40 PhD students and receives funding from the MRC. He is a member of the MRC Centre for Transplantation and Biomedical Research Centre. His research explores the genetic interactions that control tooth development together with the biology and applications of dental stem cells.

In 2004 he was awarded the Craniofacial Biology Research Award by the International Association for Dental Research in recognition of his contribution to the understanding of how teeth develop and in 2006 his paper "Stem cell-based tissue engineering of teeth" received the William J Gies award for best publication in *Biomaterials and Bioengineering* from the same organisation.

## Chapter 13



**Ravi Maddipati** is a Gastroenterology fellow in Dr. Ben Stanger's Laboratory at the University of Pennsylvania. Dr. Maddipati received his degrees in computer and biomedical engineering from the University of Michigan. He went on to obtain his MD at Boston University. During his time there he completed an HHMI research fellowship at the NIH in Dr. Richard Siegel's lab studying TNF receptor mediated auto-inflammatory disorders.

After completing his Internal Medicine training at Massachusetts General Hospital, he went to the University of Pennsylvania to pursue a clinical fellowship in Gastroenterology. Currently, his research interests center on understanding organ size regulation and its role in cancer development.



**Ben Stanger** grew up in the Lower East Side of New York and took his first biochemistry class as a student at Stuyvesant High School. He majored in biology at MIT where he worked with David Housman, helping to assemble some of the first linkage maps of the human genome. He then pursued an MD-PhD at Harvard Medical School and earned his PhD in Phil Leder's

laboratory studying the molecular mechanisms of apoptosis. After completing clinical training in Internal Medicine at UCSF and Clinical Gastroenterology at the Massachusetts General Hospital, he did a postdoctoral fellowship with Doug Melton where he became interested in organogenesis and cancer. Since starting at the University of Pennsylvania in 2006, Ben's laboratory has used the mouse pancreas and liver as model systems to understand the cellular and molecular mechanisms underlying tissue growth, regeneration, and metastasis.

## Chapter 14



**Dr. Mitsuyasu Hasebe** got Ph.D. at the University of Tokyo in 1992 and is a professor in the laboratory of Evolutionary biology, National Institute for Basic Biology, Okazaki, Japan since 2000. His group is working on the molecular mechanisms of stem cell regeneration in the moss *Physcomitrella patens* as well as the evolution of stem cell formation in the life cycle of land plants. Lab web pages are <http://www.nibb.ac.jp/evodevo> and <http://moss.nibb.ac.jp>.



**Philip Benfey** is a HHMI/GBMF Investigator and the Paul Kramer Professor of Biology at Duke University. Dr. Benfey received his PhD from Harvard University and a DEUG (Diplomé Etudes Universitaires Générales) from the University of Paris. He is a fellow of the American Association for the Advancement of Science and a member of the US National Academy of Sciences. His research focuses on plant developmental genetics and

genomics. For more information about Dr. Benfey and his research group, please see the lab webpage at <https://sites.duke.edu/benfey/>.



**Erin Sparks** is a post-doctoral scholar in Dr. Philip Benfey's laboratory at Duke University. Dr. Sparks received a B.S. in Biomedical Engineering from Northwestern University and a PhD in Cell and Developmental Biology from Vanderbilt University. Her research interests center on genome-wide approaches to understand cell fate progression and organogenesis.



**Akihiro Imai** received Ph.D. in plant biology from Hokkaido University, Japan, in 2006. He has been studying on the molecular mechanism of the stem cell formation in the moss *Physcomitrella patens* as a post-doctoral scholar in Dr. Philip Benfey's laboratory at Duke University from 2011 to 2013 and in Dr. Mitsuyasu Hasebe's laboratory at National Institute for Basic Biology, Japan, since 2013.

## Chapter 15



**Teresa Adell** performed her PhD, in the IMIM (Barcelona), where she studied the mechanisms that control the differentiation of the acinar versus the ductal cells, the two main cellular types that compose the exocrine pancreas. During her first post-doc, in the Johannes-Gutenberg University of Mainz (Germany), he moved from the human model to most basal animal, the sponges, from which she identified for the first time

several conserved developmental genes. Since 2005 she incorporated in E. Saló's group (University of Barcelona) where she started her current line of research, focused on the study of the role of the Wnt signaling pathway during planarians regeneration, homeostasis and also during embryonic

development. Her main interest is to understand the molecular mechanisms which instruct cell proliferation and fate during development.



**Francesc Cebrià** has dedicated his whole scientific career to the study of regeneration in freshwater planarians. After completion of his PhD at the Department of Genetics of the University of Barcelona, he moved first to Japan and then to USA as a post-doctoral fellow where he worked mainly on planarian CNS regeneration. Since 2008 he leads his own laboratory in Barcelona, where he is also professor at the Department of Genetics. His current research interests focus on characterizing the function of the EGFR signalling pathway on neoblast biology and neurogenesis. Also, he is interested in determining the role of the nervous system in controlling the process of planarian regeneration.



**Prof. Emili Saló.** 1975–1984 PhD, Department of Genetics, University of Barcelona, Spain 1984–86 Post-doctoral fellow, Biozentrum, University of Basel, Switzerland 1990 Group Leader at the Department of Genetics Of university of Barcelona, and Institute of Biomedicine of University of Barcelona (IBUB), Spain 2004–08 and since 2012, Head of the Department of Genetics, University of Barcelona Freshwater planarians is my model to study regeneration. I use the tools of molecular cell biology and functional genomics to address several major biological problems related to stem cells proliferation and differentiation. We also study axial polarity, pattern formation and pattern remodelling during regeneration and homeostasis.

## Chapter 16



**Katy L. Lawson** received a B.A. with honors in Biology and English Literature from Concordia University in Portland, Oregon (2009) and is currently pursuing a doctorate in medicine at Oregon Health & Science University. In the two years preceding medical school, Katy worked in Leonard Zon's lab at Boston Children's Hospital studying hematopoietic stem cell development and migration in the zebrafish model. In 2013, she was awarded an American Society of Hematology HONORS Award and is studying microRNA expression in Fanconi anemia stem cells in the lab of Peter Kurre at OHSU. In her free time, Katy intends to stay actively involved in hematological research while enjoying the beautiful outdoors of the Pacific Northwest.



**Leonard I. Zon (MD)** is Grousbeck Professor of Pediatric Medicine, Harvard Medical School; Investigator, Howard Hughes Medical Institute; and Director of the Stem Cell Program, Children's Hospital Boston.

He received a B.S. in chemistry from Muhlenberg College (1979) and an M.D. from Jefferson Medical College (1983). He did an Internal Medicine residency at New England Deaconess Hospital (1986) and a Medical Oncology

Fellowship at Dana-Farber Cancer Institute (1989). His postdoctoral research was in Stuart Orkin's laboratory (1990). Dr. Zon is internationally recognized for his pioneering work in stem cell biology and cancer genetics. He has been the pre-eminent figure in establishing the zebrafish as an invaluable genetic model for the study of blood and hematopoietic development. He is founder and former President, International Society for Stem Cell Research; Executive Committee Chair, Harvard Stem Cell Institute; and in 2005, completed a term as President of the American Society for Clinical Investigation and was elected to the Institute of Medicine of the National Academies. Dr. Zon was elected to the American Academy of Arts & Sciences (2008) and awarded the E. Donnell Thomas Lecture and Prize from American Society of Hematology (2010).

## Chapter 17



**Dr. Hans Joachim Rolf** studied at the Georg-August-University in Göttingen Geology, Biology, Education Science, Journalism and Communication Science. He worked for five years as a student trainee at the *German Collection of Microorganisms* in Göttingen. Subsequently, he entered the *Study Group of Comparative Hormone Physiology* at the Zoological Institute of the University of Göttingen working with fallow deer. His diploma thesis

has dealt with the influence of sex hormones on the annual antler regeneration in deer. After receiving his diploma in Zoology, he continued his work in the context of a doctoral research study and received his PhD in 1990. During the following years he worked at the University of Tübingen (Germany) as an assistant professor at the *Institute of Brain Research*. In 1996 he returned to Göttingen and focused his research interests on "basic research using the model of annual regenerating deer antlers". He built up a cell culture lab at the *Orthopedic Hospital* of the University Medicine (UMG) in Göttingen and since October 2000 he changed as Project Manager/Head of the Laboratory to the *Research Group of Experimental Osteology* at the Dept. of Maxillofacial Surgery. His main areas of research are stem cells, pluripotency factors, tissue regeneration, experimental osteology and deer antlers.



**K. Günter Wiese** is a professor of Maxillofacial Surgery at Georg-August-University Göttingen, Germany. He graduated with a degree in medicine and dental medicine from the Göttingen University, and completed a research fellowship at Max-Planck-Institute for Experimental Medicine, Department of Physiology with a PhD in Medicine. After a clinical training in Anaesthesiology and Surgery at different hospitals he finished his training in Craniomaxillofacial Surgery at the Göttingen University Hospital. Confronted with tissue and bone defects in his patients he started doing research on tissue engineering, bone physiology and bone response to implants. Currently, his main research interests are focused on stem cells and tumour biology.

## Chapter 18



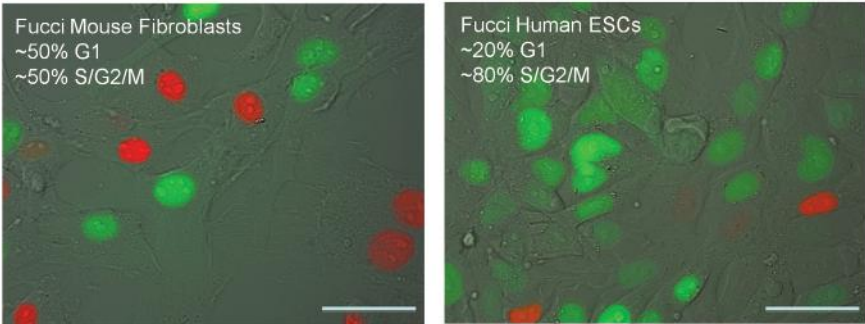
**Konstantinos Anastasiadis** was born in 1962 in Thessaloniki, Greece. He studied animal sciences in Thessaloniki followed by post-graduate studies in Montpellier, France. Hethen moved to TU Munich in Germany for his doctoral thesis and worked on the topic of mouse transgenesis. From 1996 till 2001 he did a postdoc in EMBL, Heidelberg, where he got familiar with gene targeting technology and he was involved in the analysis of different mouse knockout models including the one for the pluripotency factor Oct4. In 2002 he moved to BIOTEC, TU Dresden and established his lab with a focus on genetic engineering. Major achievements are transposon mediated BAC transgenesesis, optimization of the tetracycline inducible system and development of site-specific recombination tools that can be used for conditional mutagenesis.

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## **Color Plate Section**

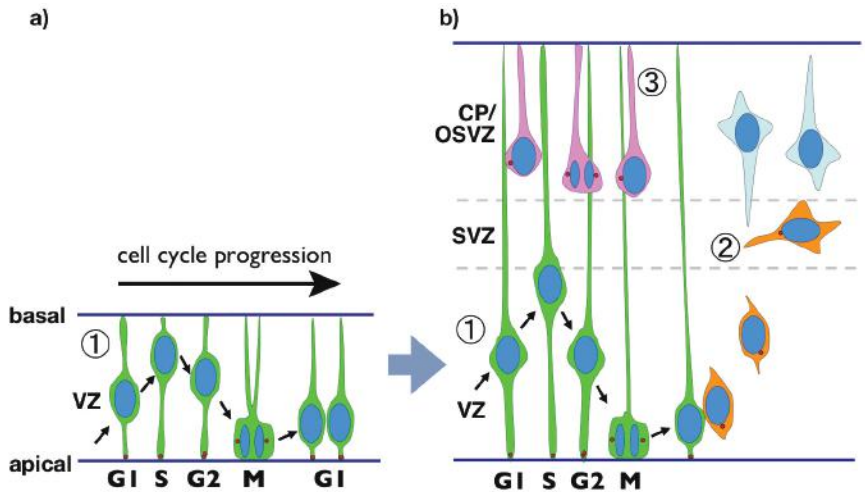


## Chapter 1



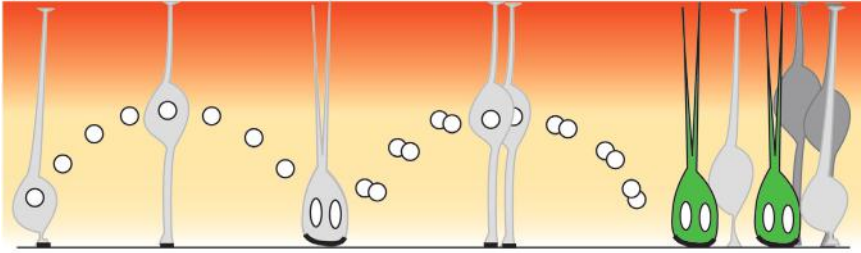
**Figure 1.3** Pictures of liveFucci cells. Mouse fibroblasts (left) and human embryonic stem cells (right) expressing Fucci reporters to identify G1/G0 (red) or S/G2/M (green) cells.

## Chapter 2

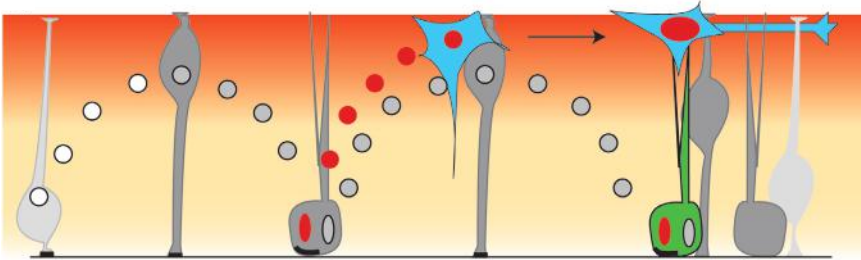


**Figure 1.** Neural progenitors in the developing mammalian cortex Drawings representing early (A) and mid/late (B) neurogenesis with neuroepithelial/radial glial cells (1), basal/intermediate (2) and OSVZ (3) progenitors. Nuclei of the former exhibit INM during the cell cycle (arrow). Each progenitor subtype can generate post-mitotic neurons (light blue). VZ=ventricular zone; SVZ=subventricular zone; OSVZ=outer subventricular zone; CP=cortical plate. Basal is up and apical down. Centrosomes are labeled in red.

A



B

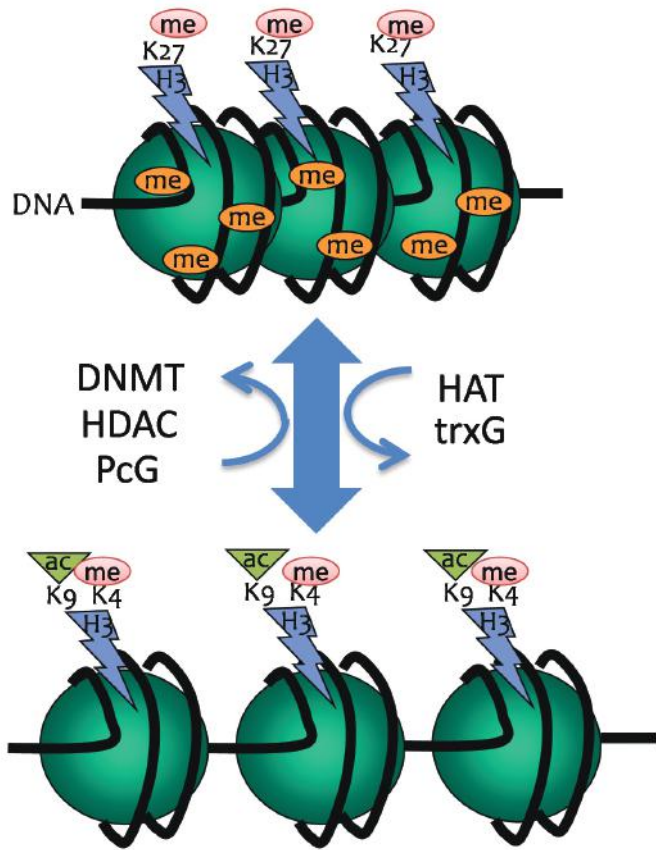


(adapted from Baye and Link, 2007 and Del Bene et al, 2008)

**Figure 3 A Model representing the complementarity of INM and asymmetric cell division regulating neurogenesis** (A) Prior to a symmetric proliferative division, the cell nucleus does not reach the neurogenic region (orange/red) at the basal surface and therefore is less likely to generate neurons, as initially proposed by Baye and Link, 2007. During symmetric divisions, the apical domain (black; with inheritance correlating with symmetric cell division) is symmetrically inherited by the two daughter cells. (B) In neurogenic divisions, cells nuclei move further basally and reach a putative neurogenic region (orange/red). This probably contributes to committing the progenitor (dark grey) to produce at least one neuron (light blue) in the next cell division. The asymmetric inheritance of factors and structures, as illustrated by asymmetric inheritance of the apical domain (black) and basal process, promotes daughter cells to adopt asymmetric fates (for example, neuron and progenitor).

### Chapter 3

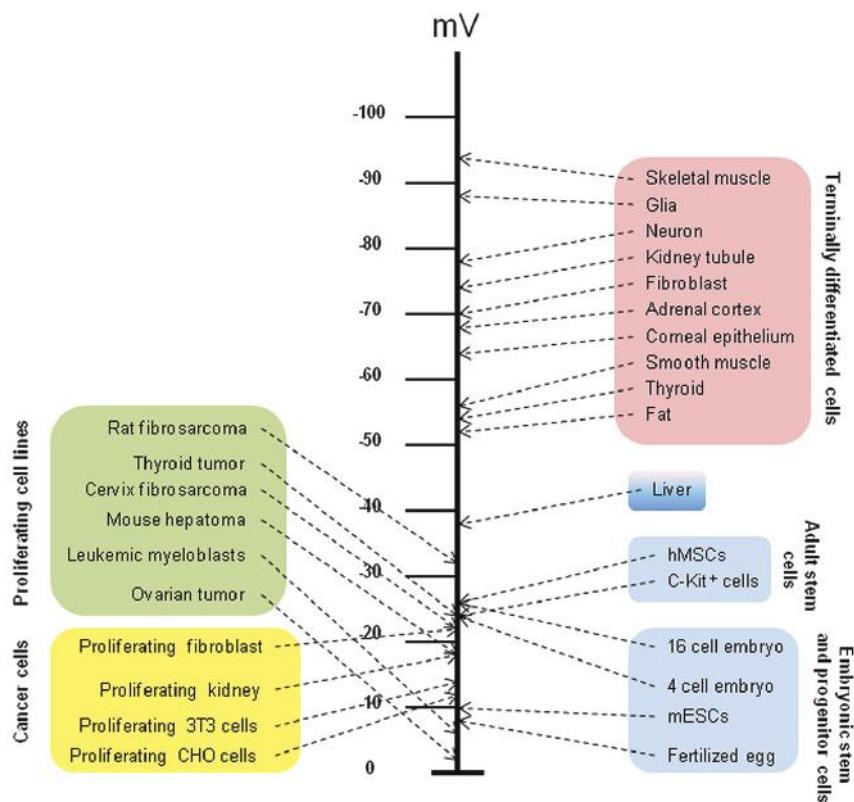
#### Transcriptionally inactive chromatin



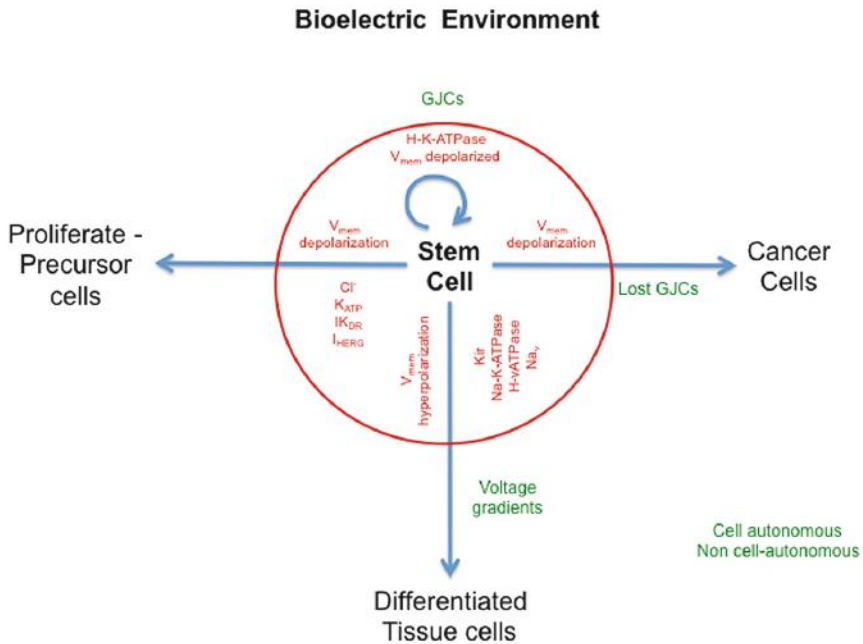
#### Transcriptionally active chromatin

**Figure 2. Reversible epigenetic switching between transcriptionally active and inactive chromatin states.** The inactive state is characterized by densely packed nucleosomes (green balls), polycomb-induced H3K27 trimethylation and HDAC-mediated deacetylation, as well as DNMT-induced DNA methylation. In contrast, the transcriptionally active state is characterized by loosely packed nucleosomes, acetylation of H3K9 and trithorax-mediated methylation of H3K4.

Chapter 5

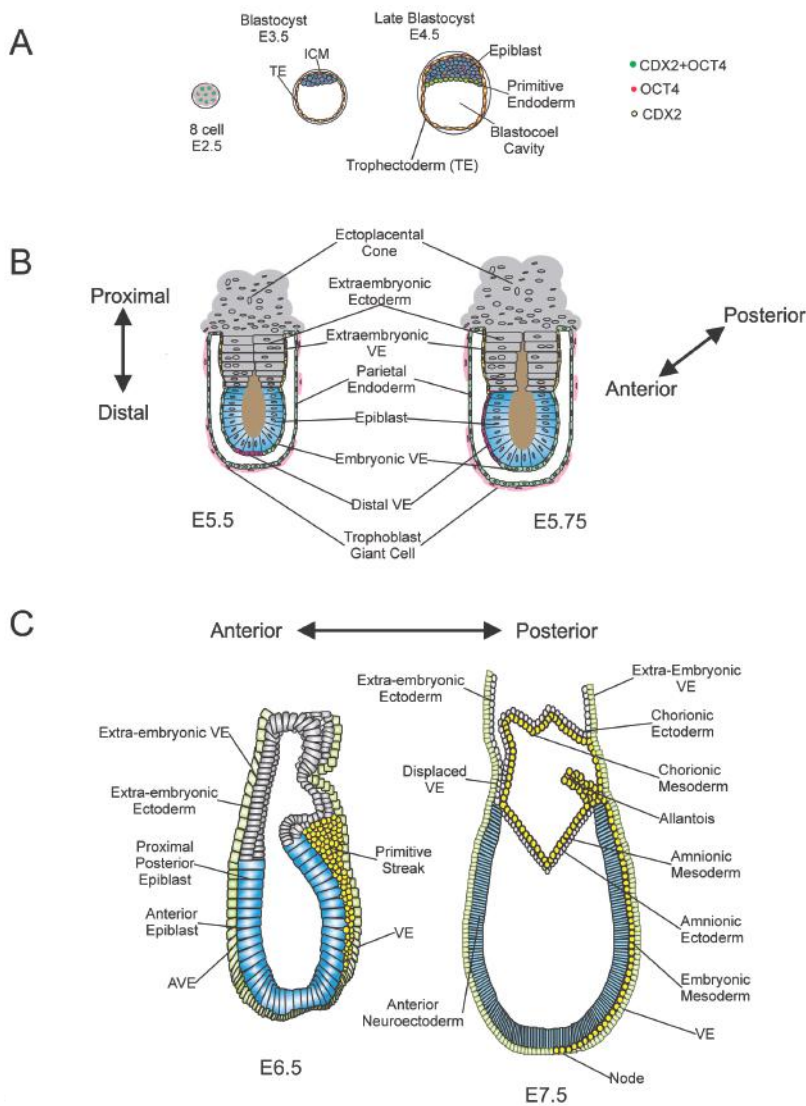


**Figure 1.  $V_{mem}$  control of stem cell maintenance and differentiation.** Physiological measurements of various cell types as documented in [modified from (Binggeli and Weinstein, 1986)]. Stem cells and highly proliferative cells have a relatively depolarized  $V_{mem}$ . However, quiescent, terminally differentiated cells are strongly polarized. A deviation from this is seen in the liver tissues which have a  $V_{mem}$  closer to the proliferative and stem cells than differentiated cells. It highlights the highly responsive progenitor cells population and high regenerative ability of liver tissues. Analogously, proliferative cancer cells also cluster with stem and highly proliferative cells. The relationship between  $V_{mem}$  and cell fate is thus a functional one, e.g., mature CNS neurons were induced to reenter cell cycle by forced depolarization (Stillwell et al., 1973) and not a secondary manifestation of cell fate (Levin, 2003).



**Figure 3. Bioelectric environment governing the direction of stem cell fate.** Bioelectric signals help control which of the four main paths any stem cell takes: stay quiescent, proliferate and give rise to progenitor or precursor cells, differentiate into specific cell type, or generate a neoplastic cancer cell population. Cell-autonomous events (changes of transcription or gating of the stem cells channels and pumps) are largely encoded by changes in  $V_{mem}$  and control proliferation/differentiation decisions. The non-cell autonomous signals like external electric fields generated by epithelia and GJC communication to nearby cells serve to coordinate stem cell activity with other cell populations in the host providing cues needed for control of cell number and type (such as positional information cues). Misregulation of either set of cues may be one source of cancer, in which normal proliferation and activity of stem cells is driven towards growth that lacks large-scale patterning information (tumors).

# Chapter 6



**Figure 1.** (A) Pre-implantation embryos, showing the 8 cell-staged embryo, and early and late stage blastocysts. In the early embryo, Oct4 and Cdx2 are co-expressed in all cells. E3.5–E4.5 blastocysts have Cdx2 and Oct4 partitioned to the TE and EPI. (B) E5.5, ICM (blue cells) and primitive endoderm formation (red cells), visceral endoderm, extra-embryonic ectoderm (black cells) and epiblast formation (blue cells). E5.75, movement of the visceral endoderm. (C) E6.5, primitive streak formation and E7.5, continued development of the primitive streak and embryonic and extra-embryonic mesoderm formation. ICM, inner cells mass; VE, visceral endoderm; AVE, anterior visceral endoderm. Diagram adapted from Lu et al. (2001).

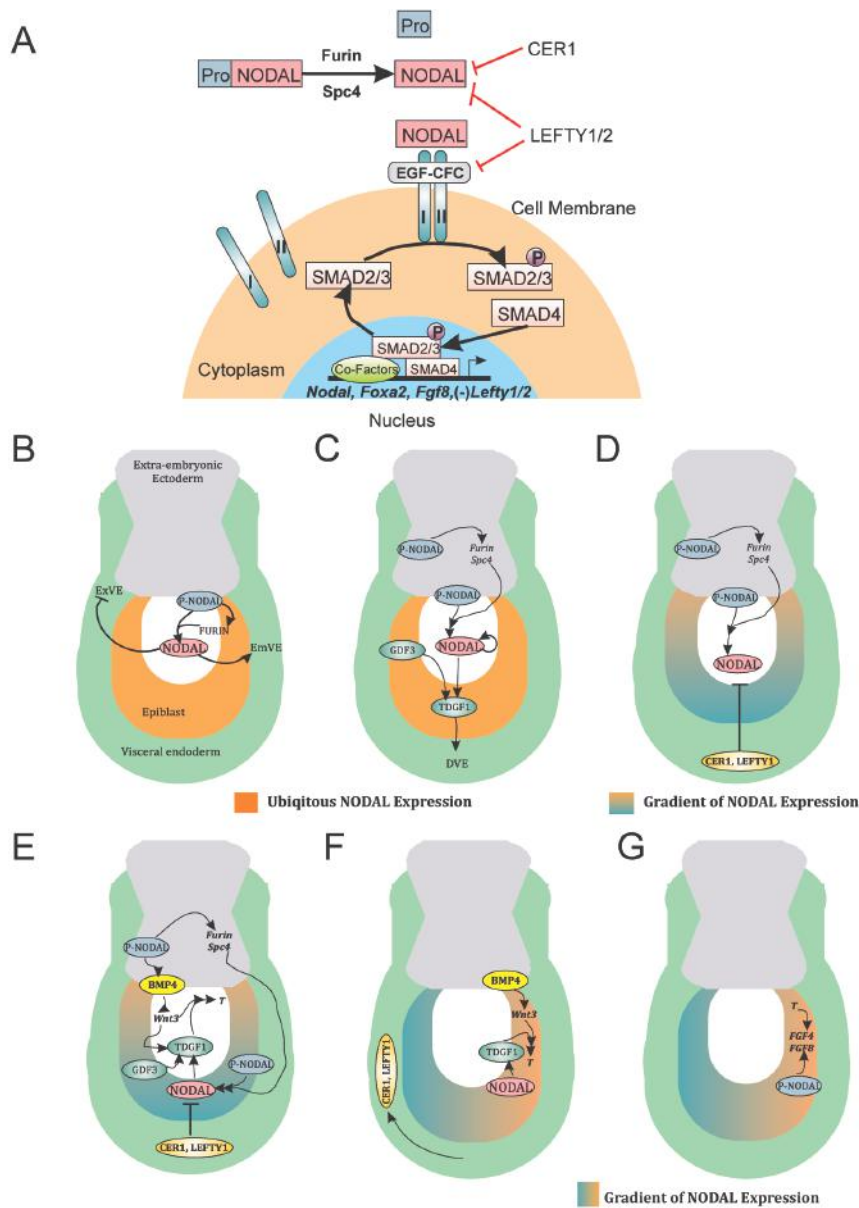
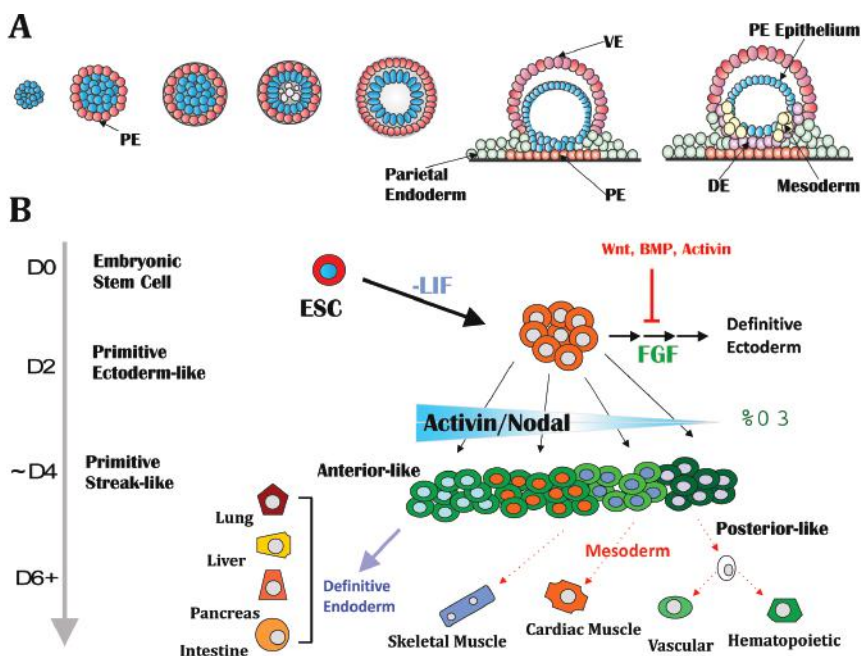


Figure 2. contd....



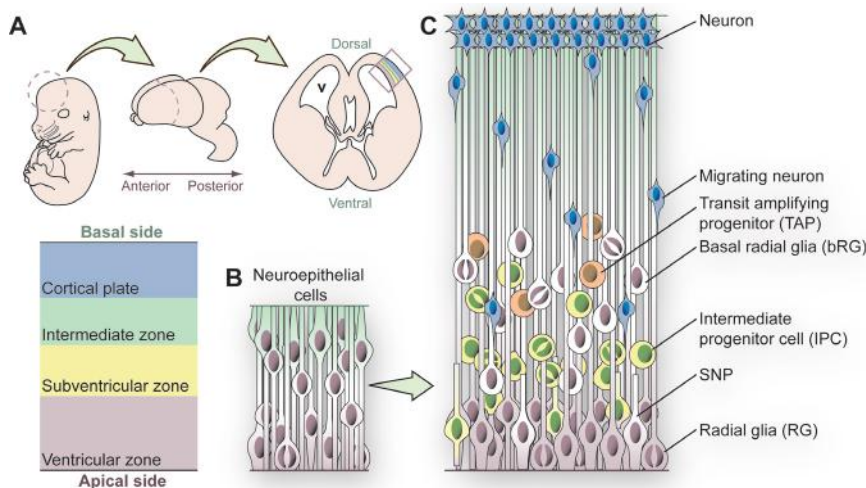
**Figure 2.** Nodal Signaling in Development. (A) The Nodal precursors (Pro-Nodal) act as partial agonists when bound to the receptors in a co-receptor independent manner. Pro-Nodal is also cleaved by the convertases Furin or SP4 to generate mature Nodal, which together with its co-receptor (TDGF1) activates the type I-II receptor complexes to phosphorylate the downstream signalling molecules Smad2/3. Activated Smads associate with co-SMAD to translocate to the nucleus where it regulates gene expression. As a regulatory mechanism, extracellular antagonists (Cer1 and Lefty1 & 2) directly bind to Nodal ligands or receptor complexes to antagonize Nodal signalling. Diagram adapted from Arnold and Robertson, 2009. (B) E5.0, Nodal precursor protein activates the transcription of *Furin* which cleaves the Nodal precursor protein to produce the processed form of Nodal. Nodal signals from the epiblast repress the expression of genes such as *Hnf4a*, *Gata4*, *Ttr* (*transferrin*) and *Furin* in the extra-embryonic visceral endoderm (ExVE). Simultaneously, in the embryonic visceral endoderm (EmVE) Nodal signals up-regulate the expression of *Lhx1*, *Fgf5*, *Fgf8*, *Bmp2*, *Otx2* and *FoxA2*. (C) E5.25, Nodal precursor protein from the epiblast acts in the extra-embryonic ectoderm to activate the transcription of *Furin* and *Spc4*, by signalling activin receptors 1B and 2A (Acv1b and Acv2a). Nodal and Gdf3, working with a co-receptor Tdgf1, specifies the distal visceral endoderm (DVE). (D). E5.5, The graded signalling of processed Nodal from the epiblast/extra-embryonic boundary is additionally antagonised by Lefty1 and Cer1 from the DVE to create the proximal-distal gradient. Diagram adapted from (Tam and Loebe 2007). (E) E6.0, Bmp4 signalling from the extra-embryonic ectoderm activates a regulatory pathway involving Wnt3 which activates *T* expression initially near the epiblast/extra-embryonic ectoderm boundary. (F) E6.25, Movement of the *Cer1* and *Lefty1*-expressing AVE to the anterior side of the embryo establishes an anterior/posterior gradient and the primitive streak forms on the posterior side of the embryo. (G) E6.5, Expression of *Fgf4* and *Fgf8*, which is downstream of T and is enhanced by the activity of the Nodal precursor protein, maintains the mesoderm and cells move through the primitive streak. Diagram adapted from Tam et al. (2007).



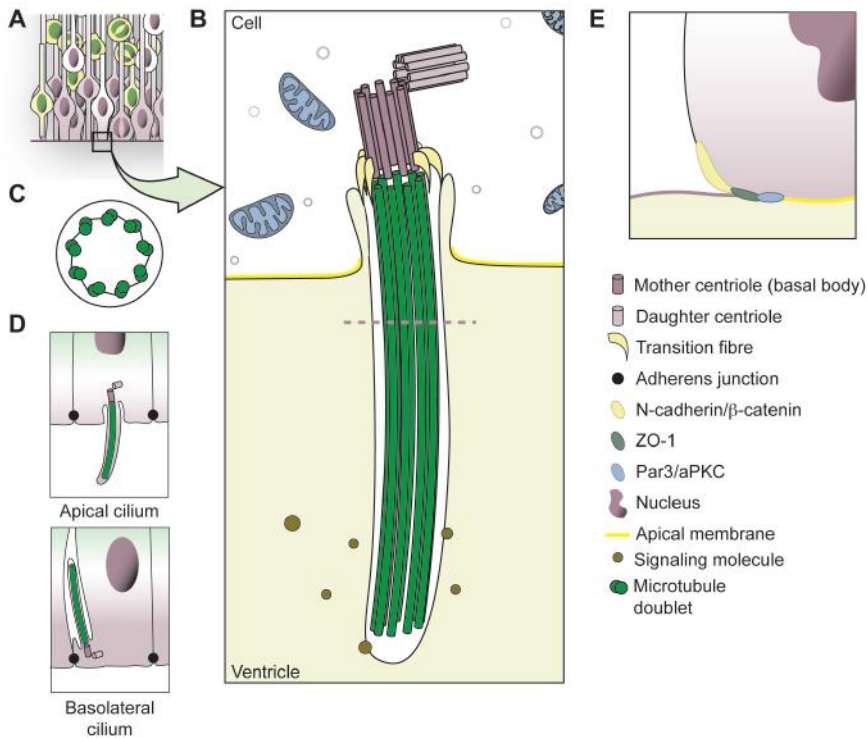


**Figure 3.** (A) Seven stages of EB formation and development. Day 1, Cdh1 mediated mESC compaction (blue cells). Days 2–4, primitive endoderm formation on the exterior of the mEB (red cells). Days 3–5, basement membrane assembly (grey). Days 4–5, primitive ectoderm epithelium formation and programmed cell death of epiblast-like cells not in contact with basement membrane. Days 5–6, continued development of primitive ectoderm epithelium, visceral ectoderm development and of cavitation completion. Day 7, mEB attachment to a plate or collagen matrix (black line) via primitive endoderm (red cells underneath the centre of the mEB) which spreads away from the mEB to form parietal endoderm (green cells). Visceral endoderm forms epithelial cysts on top of the central cell mass of the mEBs (red cells above centre of mEB). Days 8+, Primitive ectoderm epithelium (blue cells) differentiate to mesodermal cells (yellow) and definitive endoderm (purple). The culture medium substitutes for the blastocoel, and the central cyst (inside primitive ectoderm epithelium) is analogous to the pro-amniotic cavity in the embryo (diagram adapted from Li et al., 2003; Rodda et al., 2002; Weitzer, 2006). (B) Probable scheme for ESC differentiation and the role of specific factors in the formation of primitive ectoderm and primary germ layers through the generation of a hypothetical primitive streak-like structure in EBs. The formation of this hypothetical primitive streak-like structure should permit posteriorization and anteriorization of cell populations. Consistent with *in vivo* development, Fgf induces neural development independent of primitive streak formation, while Wnt, Bmp and activin are antagonistic. Bmp4 also functions to induce posterior mesoderm, and as in the primitive streak, Nodal/Activin signalling is concentration dependent. This figure has been adapted from (Perino et al., 2008).

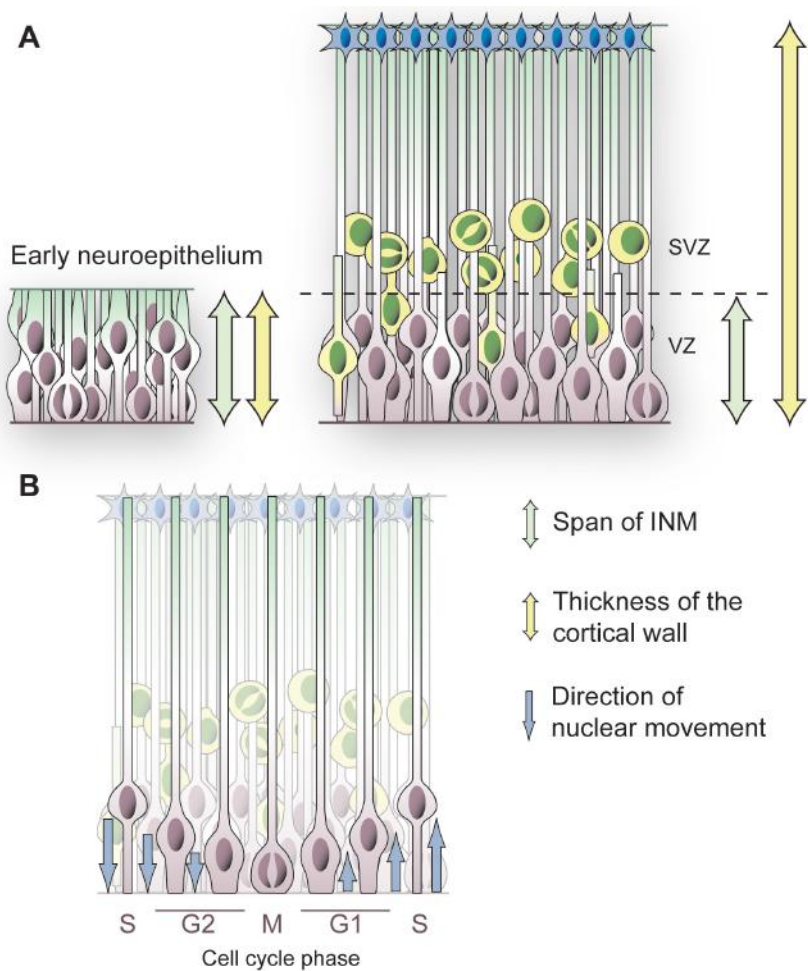
# Chapter 8



**Figure 1. Development of the mammalian neocortex.** (A) Schematic of the mouse E14 embryo and its brain. Dashed line in the brain (middle) represents the position of the coronal section (right). The violet rectangle marked on the coronal section is shown in greater detail in C. This image shows different zones in the developing neocortex: germinal zones (violet and yellow), intermediate zone and the cortical plate where the newborn neurons settle. V=ventricle. (B) Scheme of the developing brain of an undefined mammalian species at a cellular level. Left—developing neocortex at an early stage, when the only cells comprising the cortical wall are NE cells. (C) Developing neocortex at an advanced stage. Different neural progenitor populations known until now are shown in various colours.

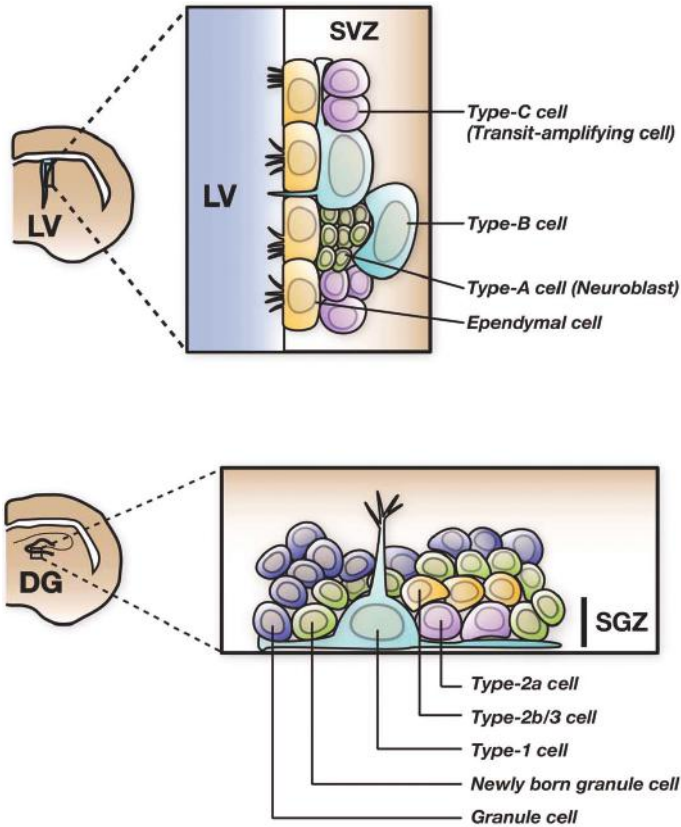


**Figure 3. Primary cilium and the apical complex of RG** (A) A portion of the cortical wall shown in greater detail in other panels. (B) The primary cilium (green), growing out of the basal body (the mother centriole) protrudes into the ventricle. (C) A section through the cilium (dashed line) reveals the arrangement of microtubule doublets. (D) Positioning of the apical cilium in an AP (top). Basolateral cilium in a soon-to-delaminate AP (bottom) (Wilsch-Bräuninger et al., 2012). (E) Schematic of the disposition of apical complex constituent in the apical portion of the AP.



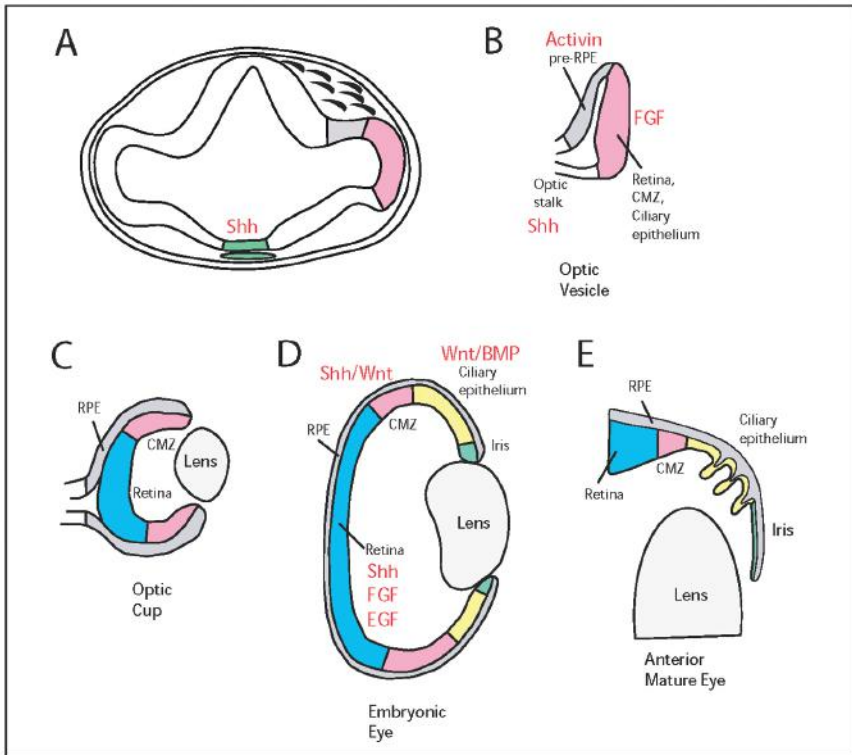
**Figure 4. Interkinetic nuclear migration of APs (A)** In the neuroepithelium at an early developmental stage INM spans the whole thickness of the cortical wall (left). After the onset of neurogenesis, when the cortical wall thickens and separates into germinal zones, the span of INM relative to the cortical wall thickness shortens (although absolutely it remains the same) (right). Colours and labels of progenitors as in Figure 1. **(B)** Direction of nuclear movement, depending on the phase of the cell cycle of an AP. Note that this scheme assumes that at least one of the daughter cells remains an AP which will continue undergoing INM.

## Chapter 9

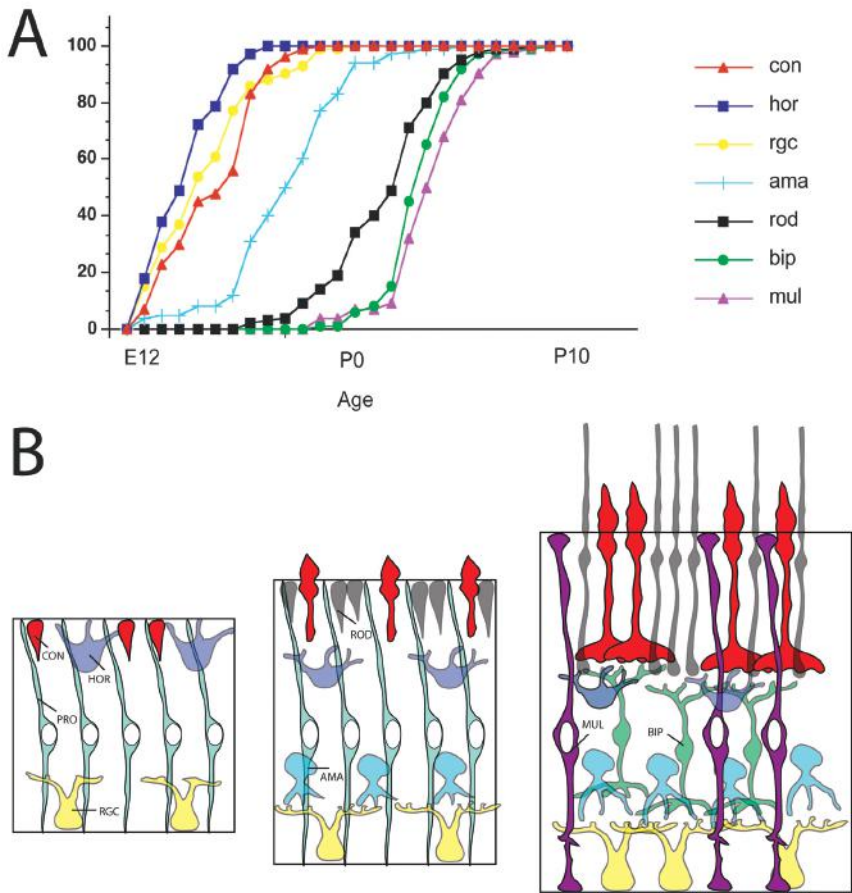


**Figure 10. NSC and neurogenesis in the adult brain.** NSCs are present in two regions of the adult brain, the subventricular zone (SVZ) of the lateral ventricles (LV) and the subgranular zone (SGZ) of the hippocampal dentate gyrus. These NSCs are mostly quiescent but occasionally divide to give rise to transit-amplifying cells, which proliferate and generate neurons. Neurons born in the SVZ migrate via the rostral migratory stream into the olfactory bulb, while neurons born in the SGZ migrate into the hippocampal dentate gyrus.

## Chapter 10

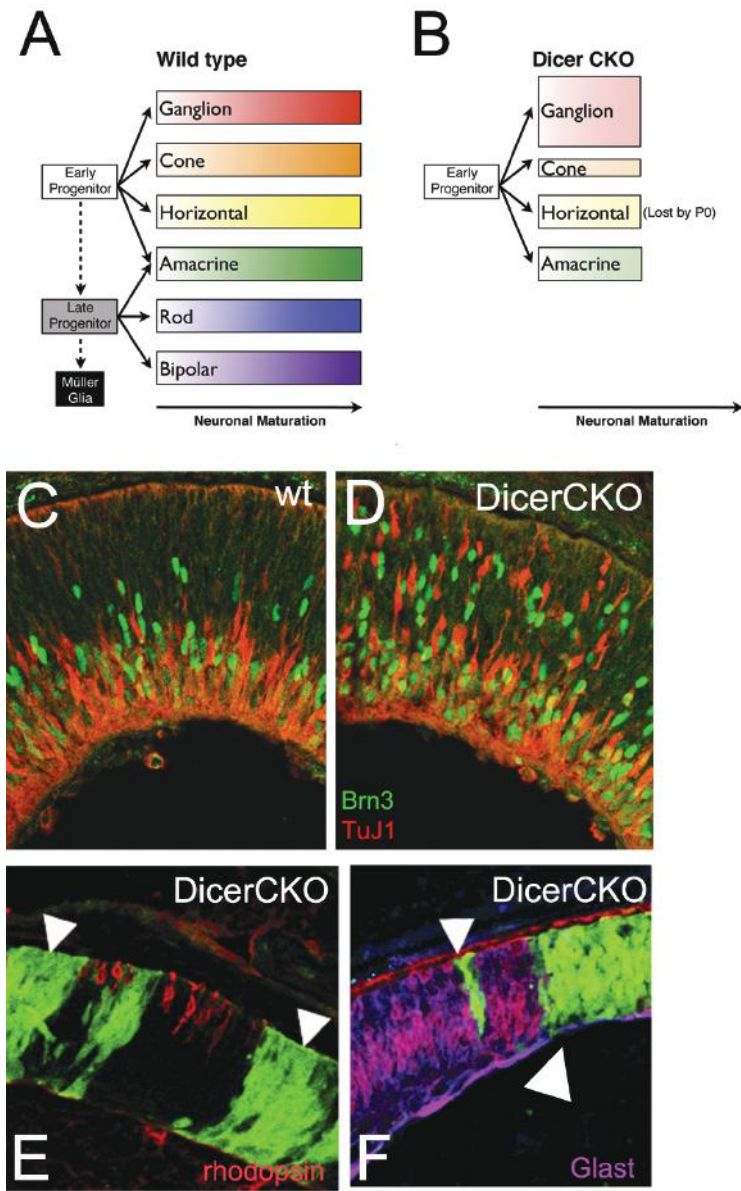


**Figure 2. Morphogenesis during eye development.** (A) The eye fields are split into two by midline sonic hedgehog signaling (Shh). (B) Several factors that are present in either the surrounding tissues or the adjacent regions of the neuroepithelium pattern the domains of the optic vesicle. (C) The optic vesicle transforms into a cup-shaped structure as the lens forms from the adjacent epithelium. (D) As the optic cup expands, the various domains of the mature eye are patterned. (E) The anterior part of the mature eye in a non-mammalian vertebrate is shown to represent the relationship among the ciliary epithelium and the retina and the stem cell zone known as the CMZ (see text for details; B-E, modified from Lamba et al., 2008).



**Figure 3. Sequential development of retinal cells.** (A) Birthdating studies of retinogenesis, showing the sequence in generation of the different types of neurons. CON=cones, HOR=horizontal cells, RGC=ganglion cells, AMA=amacrine cells, ROD=rod photoreceptors, BIP=bipolar cells, MUL=Müller glia. (B) The progenitor cells (light blue) span the retina at early developmental stages and only a few types of neurons have been generated (RGC, CON, HOR). As development proceeds (middle), new cells are added into the existing structure and the retina becomes thicker with amacrine cells (AMA) and rods (ROD). In the last stage of development, bipolar cells and Müller glia are added (right panel).

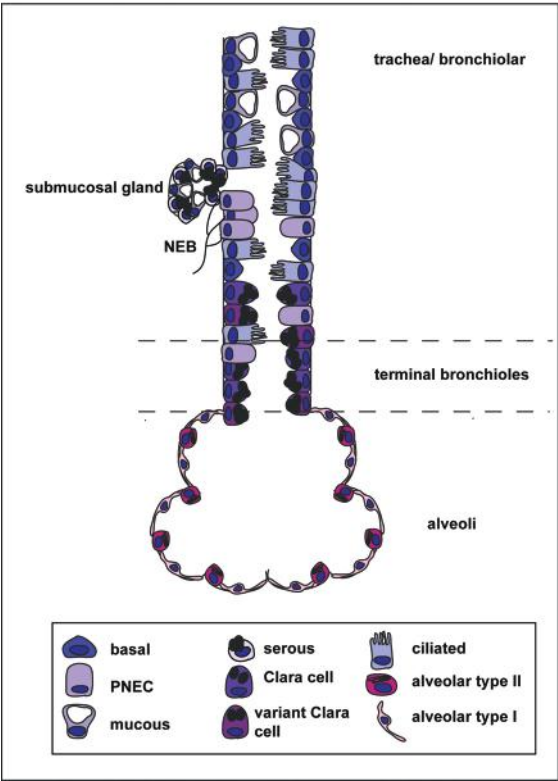




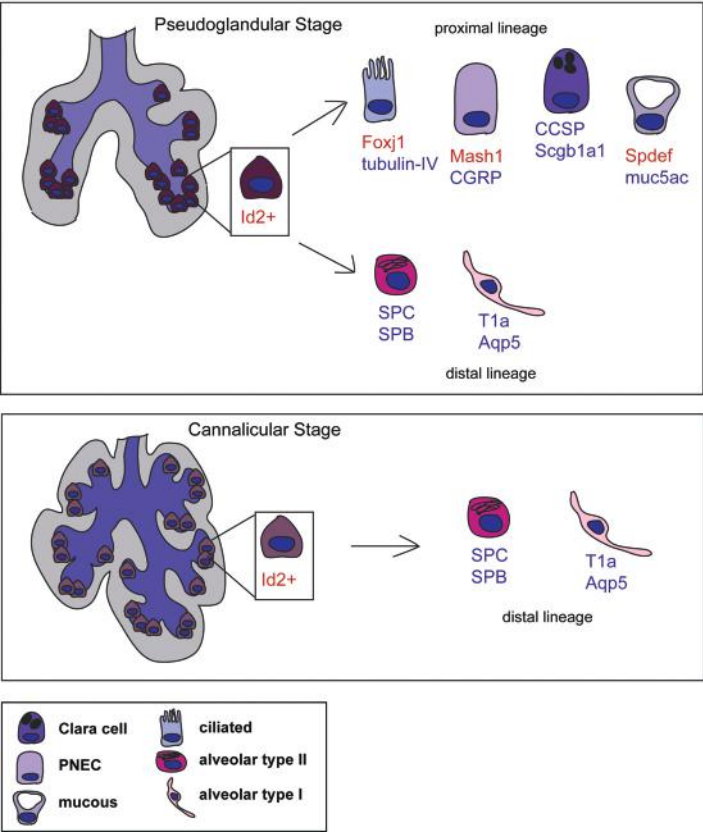
**Figure 5. MicroRNAs control the timing of retinal development.** Sequential production of retinal cell types and effects of miRNA deletions are shown (**A** vs. **B**). Conditional knockout of Dicer led to retinal ganglion cells being produced in excess (**C** vs. **D**) and rod photoreceptors (E) and Muller glia (F) being depleted in the affected regions (green). Markers used and colors are indicated in each panel (A and B modified from Georgi and Reh, 2011).



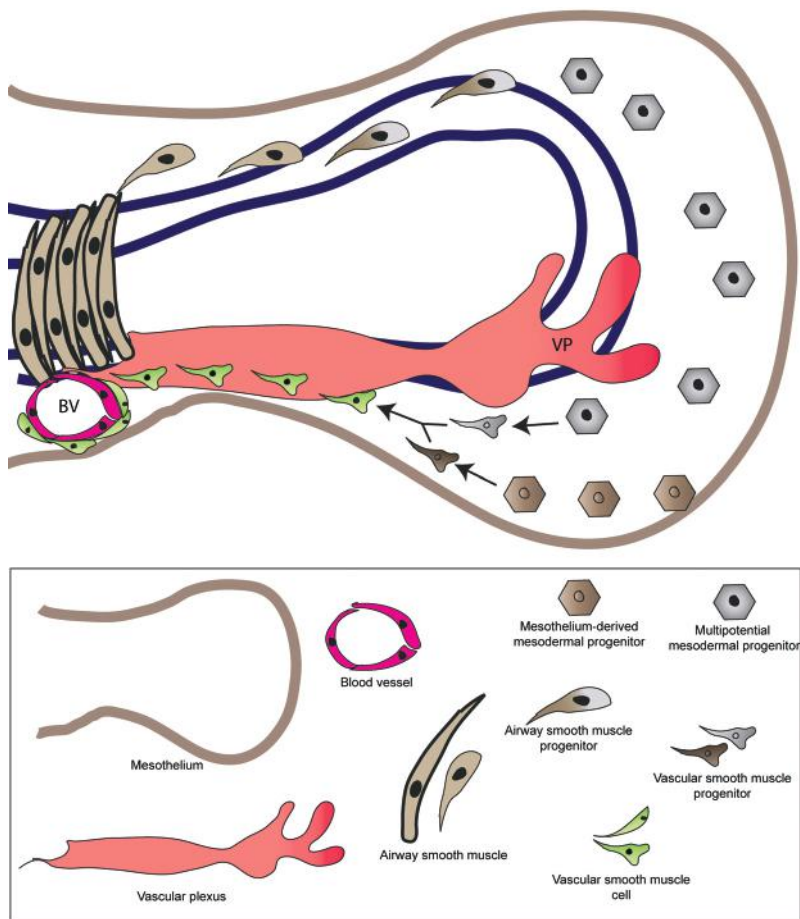
# Chapter 11



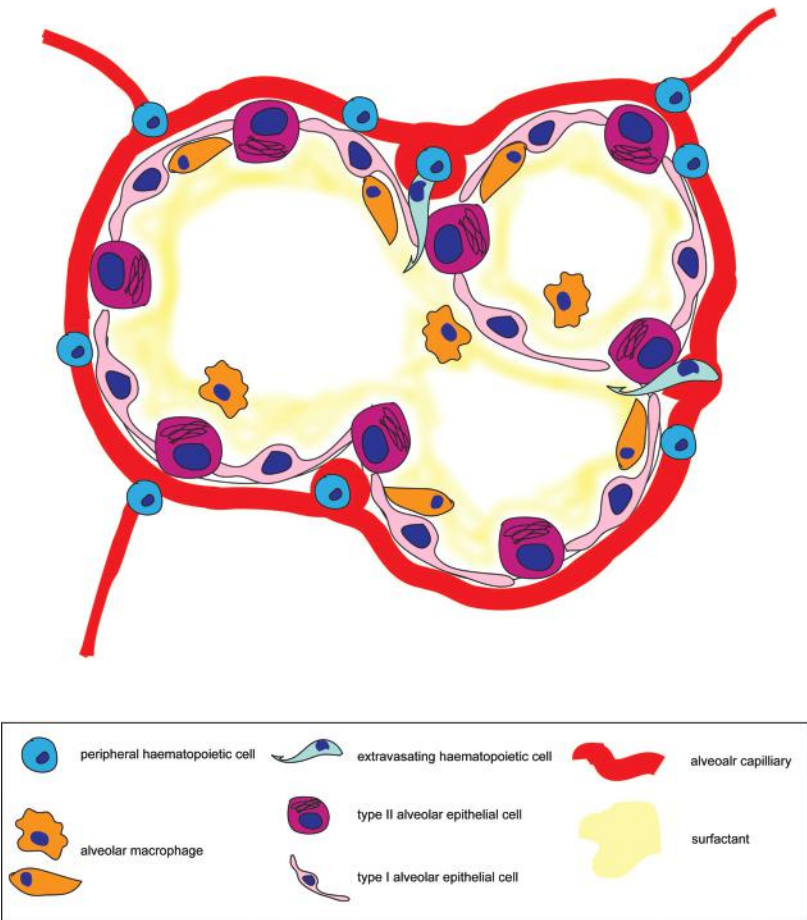
**Figure 1. Airway epithelial cells of the lung.** The tracheobronchial region of the conducting airways is lined with a pseudostratified columnar epithelium containing basal, ciliated, mucous, and occasional Clara cells. Submucosal glands lie in close proximity to neuroepithelial bodies (NEB) and serous and mucous cells. The bronchioles and terminal bronchioles are lined primarily with Clara cells, with occasional pulmonary neuroendocrine cells (PNEC), basal and ciliated cells. This area of the lung transits from a simple columnar to a low columnar/cuboidal epithelium. The alveoli are lined with very flat alveolar type I cells with a basal membrane fused to adjacent capillaries as well as cuboidal type II cells, which facilitates gas exchange. The conducting airway is depicted in blue and purple, while the gas exchange portion is shown in red and pink.



**Figure 2. Multipotent Id2<sup>+</sup> distal tip cells acts as an embryonic progenitor.** During the pseudoglandular phase of lung development, Id2<sup>+</sup> distal tip cells can contribute to the proximal and distal lineage (top). As the lung progresses into the cannalicular phase, Id2<sup>+</sup> cells become lineage restricted and only contribute to cells found in the distal lung (bottom). The adult lung does not appear to retain this multipotent population, indicating the Id2<sup>+</sup> cells only act as embryonic progenitors. The airway (blue) and surrounding mesenchyme (gray) are depicted.

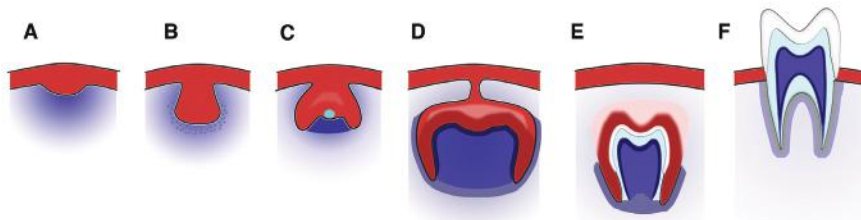


**Figure 3. Mesoderm-derived progenitors during the pseudoglandular phase of lung development.** At the distal tip multipotential mesodermal progenitors (grey hexagons) can give rise to both vascular smooth muscle progenitors (brown hexagons) near the vascular plexus (VP) or airway smooth muscle progenitors (brown & grey drops; upper portion of the figure). Fully differentiated vascular smooth muscle cells (green) arise from either mesothelium (brown hexagons) or mesodermal progenitors. These differentiated vascular smooth muscle cells surround the blood vessels (BV). Alternatively, mesodermal progenitors give rise to airway smooth muscle progenitors (top). Progenitors develop into the airway smooth muscle cells (brown vertical elongated structures) surrounding the developing airway.

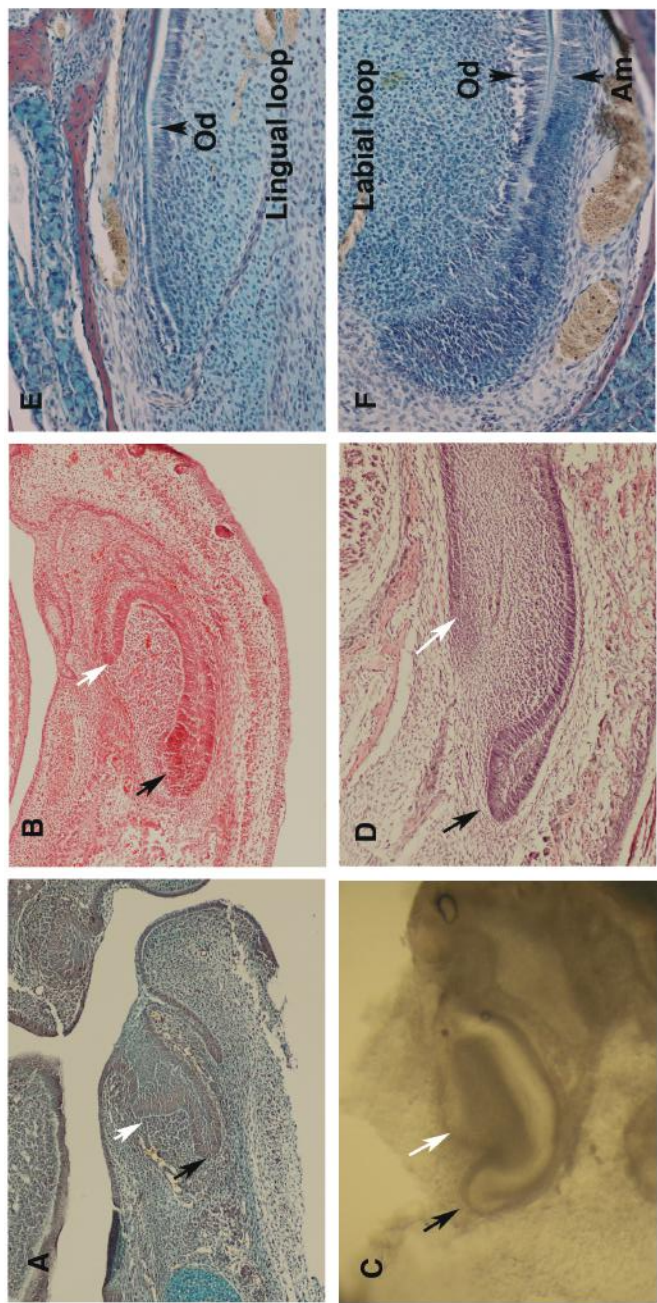


**Figure 4. Macrophages in the alveolar space.** The alveoli are saccular structures that are highly vascularized (red) with close contact amongst the alveolar capillaries and the epithelial cells (pink & purple). Hematopoietic cells (blue) circulate freely in the blood and some may extravasate into the alveoli (turquoise) to develop locally into alveolar macrophages (orange). It is presumed that alveolar macrophages reside within the lipophilic surfactant layer (yellow) and are mobile on the epithelial layer, however, some cells may not be in direct contact with the epithelial cells.

## Chapter 12

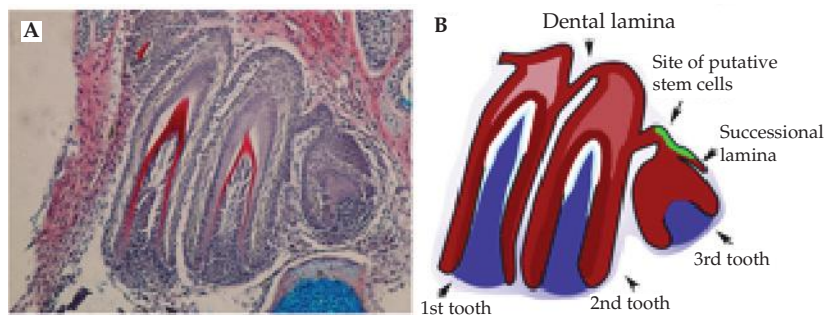


**Figure 1.** Stages of mouse molar development. (A) Thickening of the oral epithelium (red). (B) Invagination of the dental epithelium to form a bud, surrounded by condensing neural crest derived mesenchyme (blue). (C) Cap stage tooth germ. Dental papilla in blue, primary enamel knot in light blue. (D) Bell stage tooth germ. The odontoblasts (dark purple) differentiate along the inner enamel epithelium, which starts to form ameloblasts. (E) Pre-eruption. Dentin (light blue) and enamel (white) form to fix the shape of the tooth. Root development is initiated. (F) Final erupted tooth. The epithelial layers of the crown are lost.



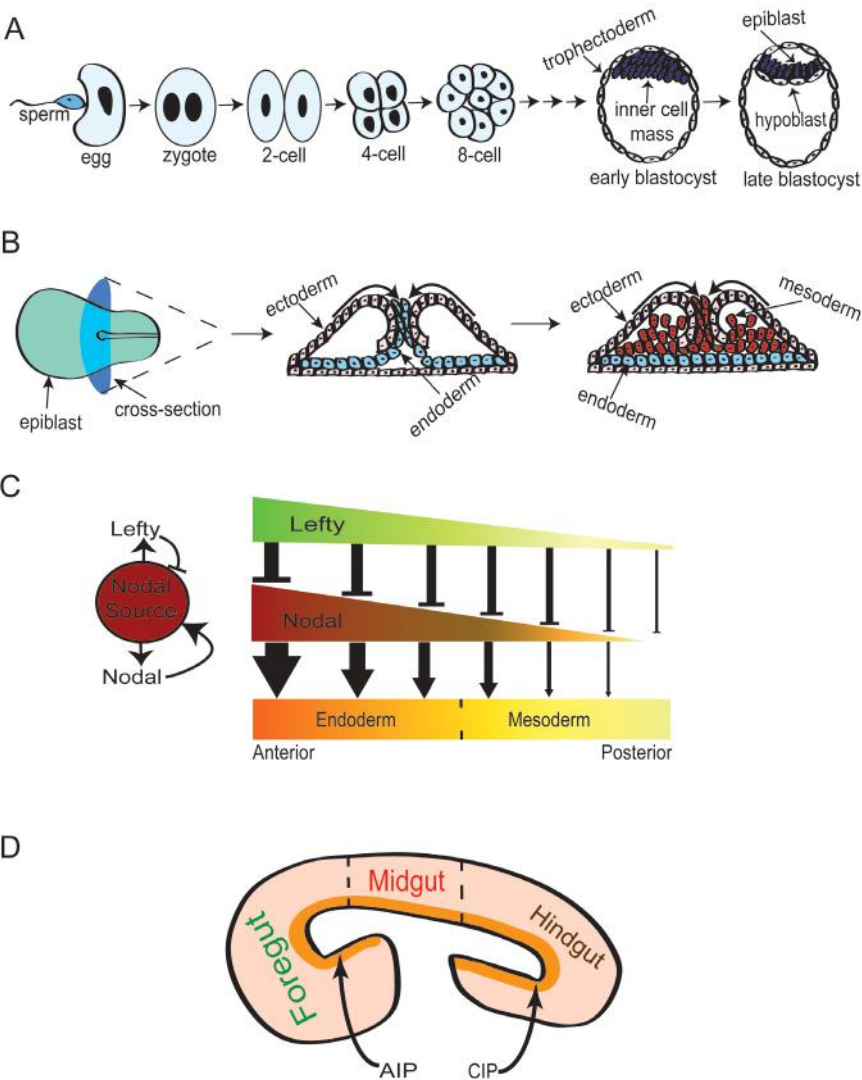
**Figure 2.** Development of the cervical loops of the mouse incisor. (A, B, D, E, F) sagittal sections. (C) Dissected incisor. (A) E14.5 cap stage tooth germ. An asymmetric development of the cervical loops is already apparent. (B) E15.5. The labial cervical loop becomes thickened in comparison to the lingual loop. (C) Dissected incisor showing the clear difference in size and shape of the loops at this stage. (D) E16.5. The labial loop has a clear layered structure with an outer basal layer and inner stellate reticulum layer. The stem cells reside in this central region. (E) The lingual loop is very thin and no ameloblasts form adjacent to the odontoblasts (Od). (F) In contrast, the labial loop is much larger and ameloblasts (Am) differentiate adjacent to the odontoblasts. White arrows in A-D indicate lingual cervical loop. Black arrows in A-D indicate labial cervical loop.





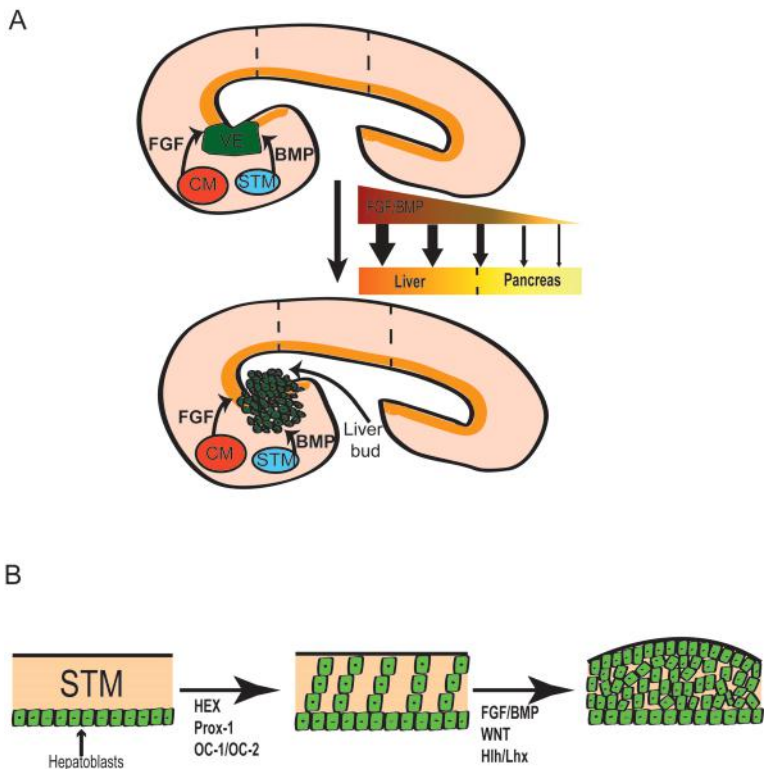
**Figure 4.** Snake replacement tooth development. **(A)** Trichrome stained section through a corn snake mandible showing formation of a series of teeth from the dental lamina. **(B)** Schematic highlighting the location of the putative stem cells (green) on the lingual side of the dental lamina, behind the successional lamina at the tip of this structure.

# Chapter 13

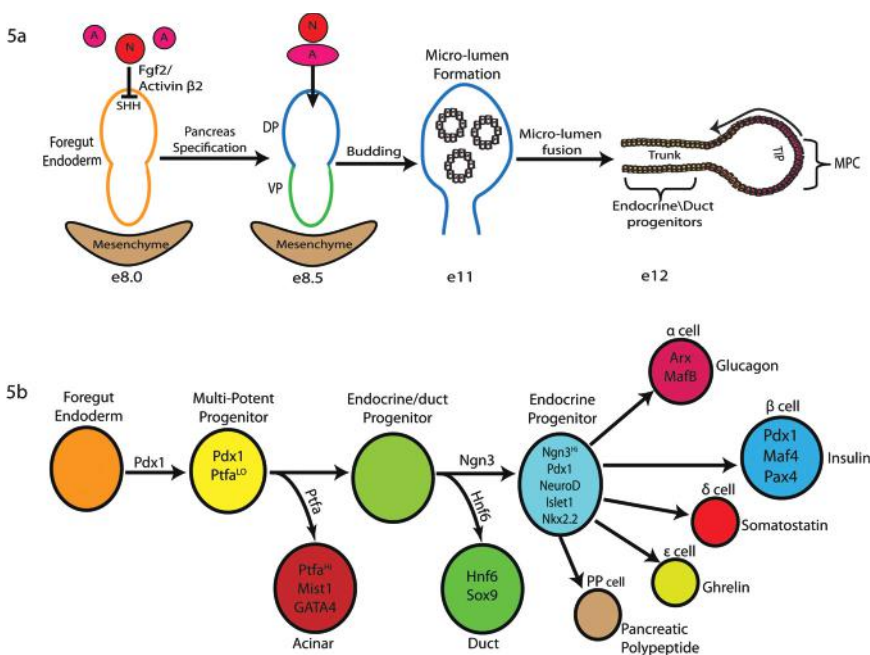


**Figure 1. overview of endoderm development.** (A) Progression from fertilized egg to blastocyst. (B) Cross-sectional view of the epiblast demonstrating migration of the three germ layers. Ectoderm (pink), Mesoderm (red), endoderm (blue). (C) Effects of Lefty and nodal on specification of endoderm and mesoderm in the epiblast. High nodal signaling overcomes left inhibition and specifies endoderm while further from the nodal source lefty inhibition of nodal has greater effect and leads to mesoderm development. (D) The segregation of the endoderm in the foregut, midgut and hindgut domains along with locations of the Anterior intestinal portal (AIP) and Caudal intestinal portal (CIP).

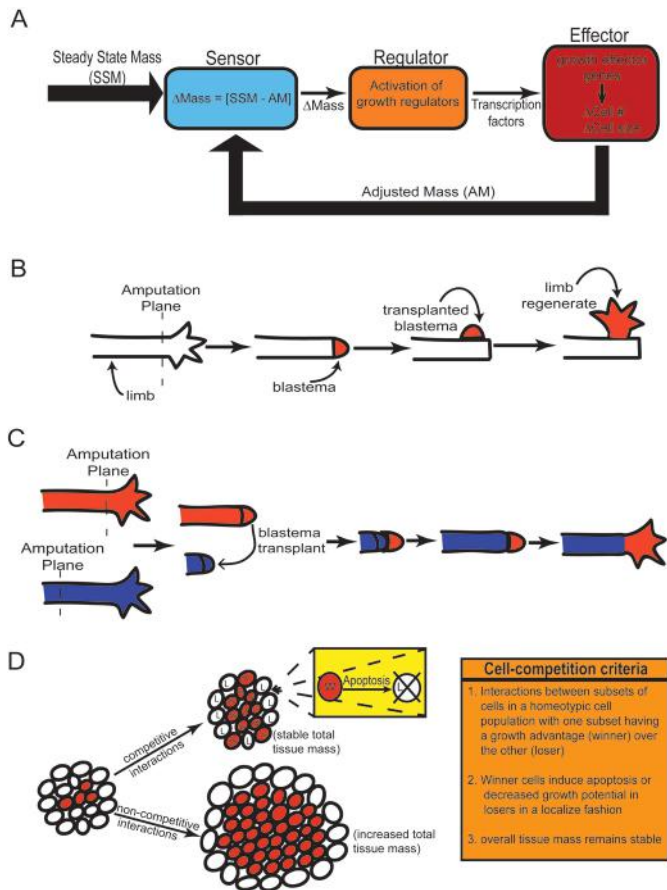




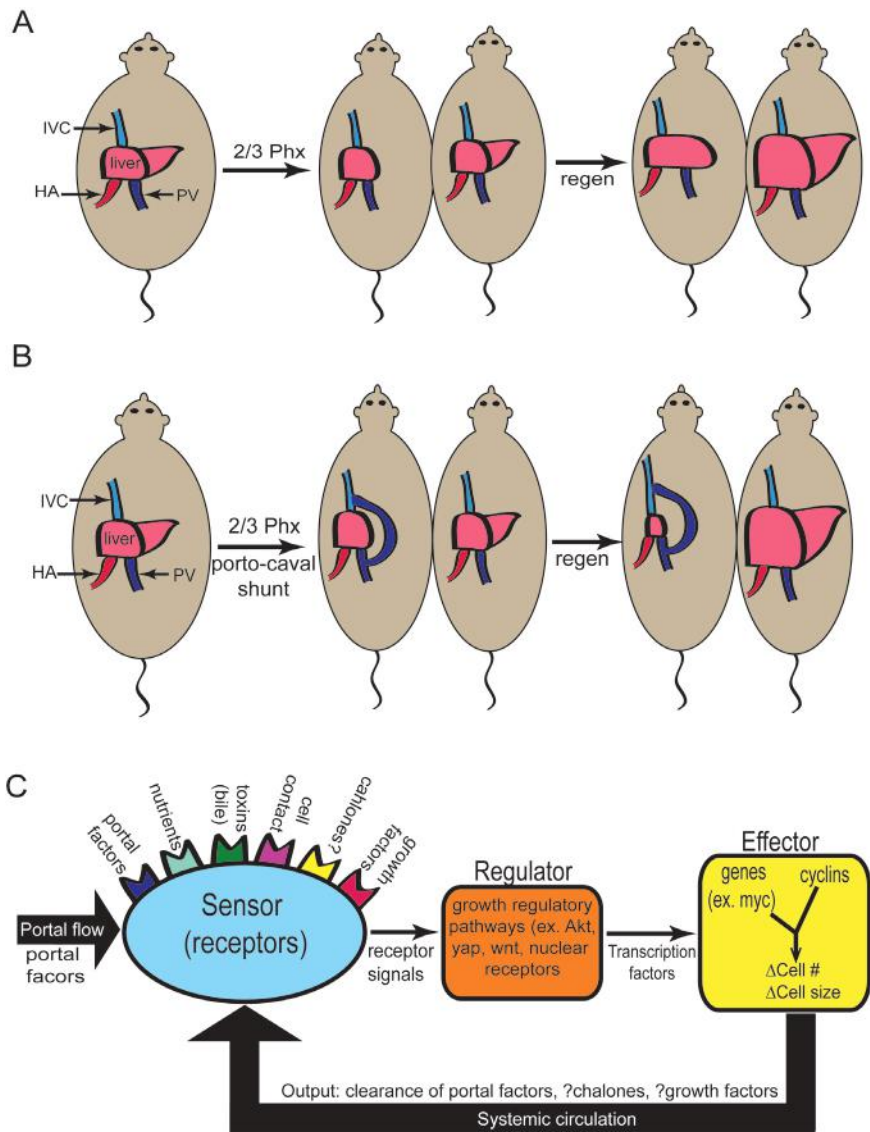
**Figure 3. liver specification and morphogenesis.** (A) Influence of Fibroblast Growth Factor (FGF) and Bone Morphogenic Protein (BMP) secreted by the Cardiac Mesoderm (CM, red) and Septum Transversum Mesenchyme (STM, blue) respectively, on the specification of Ventral Endoderm (VE) into liver and pancreas with formation of the nascent liver bud and hepatoblasts (dark green cells) in regions of high FGF and BMP signaling. (B) Migration of the hepatoblasts (green cells) into the STM (pink) to form the liver bud.



**Figure 5. mouse pancreas organogenesis.** (A) Schematic representation of pancreatic epithelial development and specification of acinar (red) and endocrine/duct (blue, green, respectively) lineages. Following fusion of microlumens and into tubular structures, formation of tip and trunk compartments occurs. Multi-potential Pancreatic Cells (MPC) along the tips take on an endocrine/ductular fate as cells migrate from tip to trunk regions. Between E13.5–14.5 the MPC become restricted to an acinar fate. (B) Transcriptional regulation of pancreatic development.  $\epsilon$  cells develop in the absence of Pax4 and Nkx 2.2 Adapted from Pan and Wright, 2011.

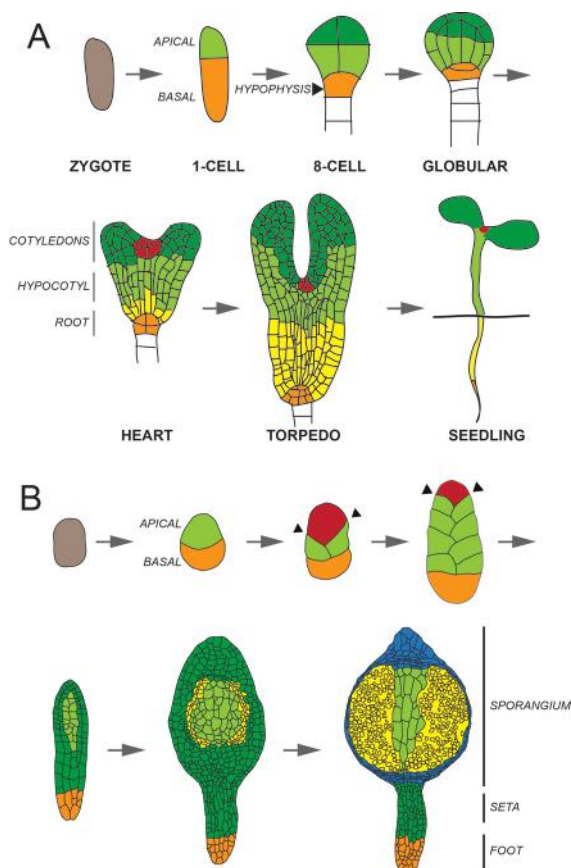


**Figure 6. principles of organ size determination.** (A) Closed loop control system model for organ size regulation. Current mass of an organ is sensed by a sensor (blue) and compared to a value indicative of necessary organ mass required to maintain homeostasis. This difference in mass triggers activation of growth regulators (Regulator, orange) which leads to changes in cell size and number (Effector, maroon) in an attempt to re-establish appropriate mass. (B) Autonomously controlled salamander limb regeneration. When the blastema (red) at the site of amputation is transplanted to another location on the limb, it only regenerates the portion of the limb distal to the site of the original amputation. (C) Regulated control of salamander limb regeneration. When the blastema (red) from one amputated limb is transplanted onto a blastema (blue) that has formed at a different amputation site, the two blastemas communicate such that the transplanted blastema delays its regeneration until proximal limb formation has occurred (D) Model of Cell competition. In competitive interactions, winner cells (red) increase in number at the expense of loser cells (white) through induction of apoptosis in the loser cells. This leads to increase in the winner:loser cell ratio but overall organ size is maintained. In non-competitive interactions the growth advantaged (red) cells increase in number but the number of wild type (white) cells does not decrease leading to an increase in overall organ size.

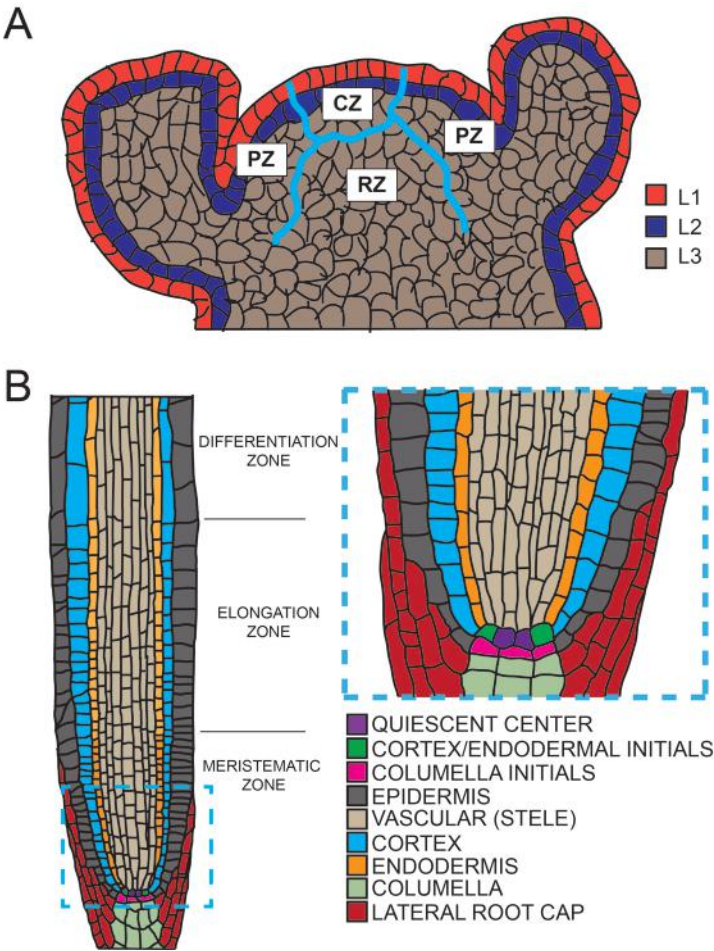


**Figure 7. regulation of liver size.** (A) Parabiosis of partial hepatectomized rat with a normal rat, leading to increased liver size of non-operated parabiotic partner. (B) Parabiosis of normal rat with a rat that has undergone partial hepatectomy and porto-caval shunt leads to increase in size of normal rat liver and decrease in size of partial hepatectomized liver. (C) Model of liver size regulation. The liver is able to detect various measures of its ability to perform normal homeostatic functions and in settings where differences are detected, size is adjusted to compensate.

## Chapter 14

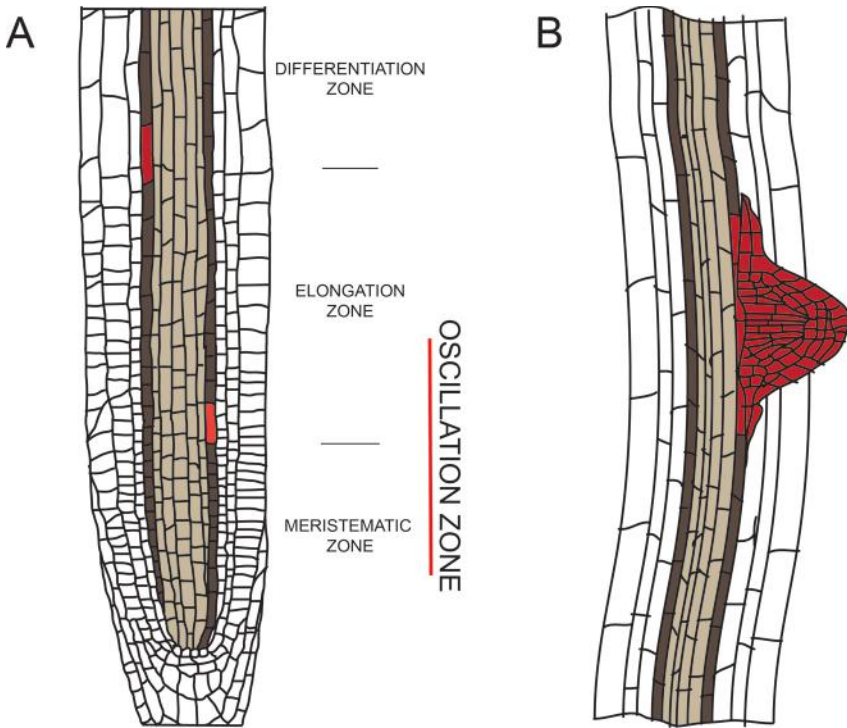


**Figure 2. Embryonic/Sporophyte Development of *Arabidopsis* and *Physcomitrella*.** (A) In *Arabidopsis* embryonic development begins with an asymmetric division of the zygote to produce an apical (light green) and basal (orange) cell in the 1-cell stage. Subsequent cell divisions of the apical cell result in an 8-cell stage (4 more cells behind what is depicted). At the 8-cell stage, the apical cells can be separated by the fates they will give rise to – dark green the cotyledons and light green the hypocotyl. In addition, the upper most cell of the basal lineage (hypophysis) is generated. The next stages are globular and heart. During the heart stage, cells fated to become the apical meristem are distinguishable (red). Additionally, the lower portion of the apical cells (yellow) and the hypophysis (orange) are distinguished as giving rise to the root. In the subsequent stage, torpedo, the cell layers are maintained to produce the embryonic seedling. Colors mark final location of shoot and root meristems, and developmental lineages. (B) *Physcomitrella* sporophyte development begins with an asymmetric division of zygote to give rise to apical (light green) and basal (orange) cell fates. Subsequent cell divisions result in a mature sporophyte consists of three parts: sporangium (capsule indicated in blue, spores indicated in yellow, and columella indicated in light green), seta (dark green), and foot (orange). Sporophyte apical cells are indicated in red. Arrowheads indicate planes of cell division. Original pictures for *Physcomitrella* drawings were provided by Dr. Keiko Sakakibara.



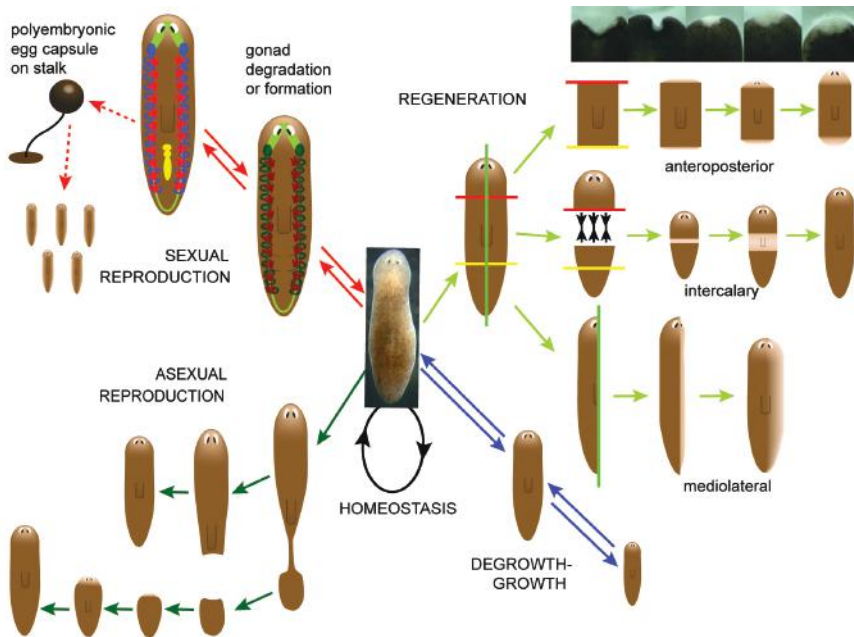
**Figure 3. *Arabidopsis* Meristems.** (A) The shoot meristem in *Arabidopsis* gives rise to all of the above ground organs. It can be divided into three layers: L1 (red), L2 (Blue), and L3 (grey) and three zones: Central Zone (CZ), Peripheral Zone (PZ) and Rib Zone (RZ). Light blue line indicates separation of zones. (B) The root meristem in *Arabidopsis* gives rise to the below ground organs. The root has radial or bilateral symmetry and can be divide into three developmental zones longitudinally beginning at the root tip: Meristematic Zone, Elongation Zone and Differentiation Zone. The Meristematic Zone contains the stem cells and niche cells, as indicated. Panel on the right is enlarged from the blue dashed box on the left.





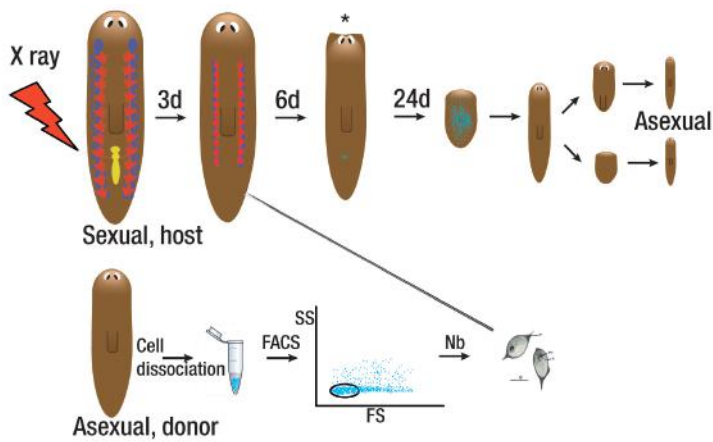
**Figure 5. Lateral Root Formation in Arabidopsis.** (A) Root diagram depicting the specification of lateral roots in Arabidopsis. Light brown indicates the stele (vascular) tissue. Dark brown indicates the pericycle cells of the root. Lateral root position is defined by an inherent time keeping mechanism of gene oscillation within an oscillation zone. Red indicates the future site of lateral root formation. (B) Lateral root emergence occurs within the differentiation zone through a series of stereotypical divisions. After emergence from the parent root, the newly formed lateral root meristem is responsible for root growth.

## Chapter 15

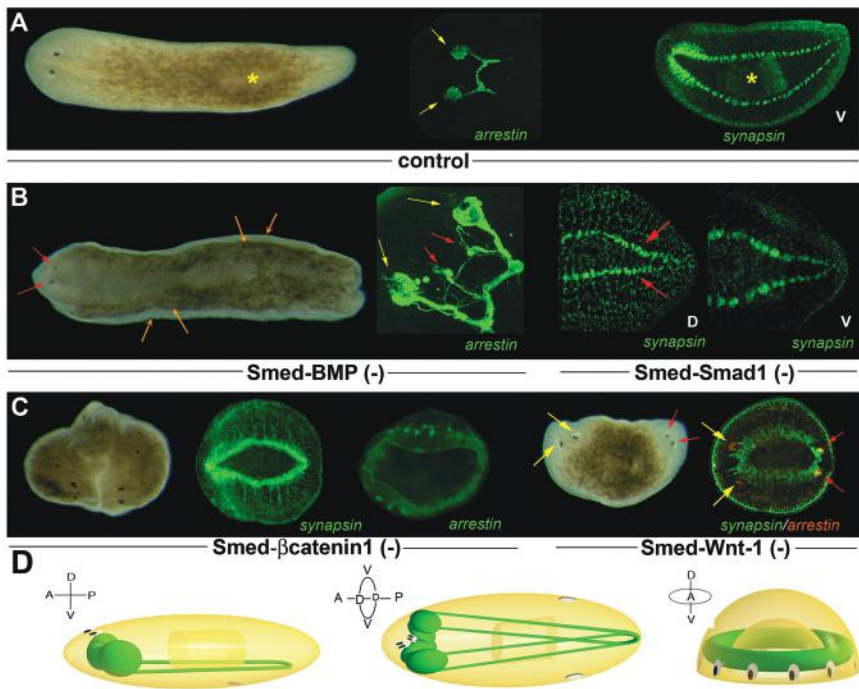


**Figure 1.** Planarian homeostasis and regeneration. **Growth–degrowth.** Planarians are very plastic, and upon starvation there is a reduction in the total body cell number (degrowth) caused by an increase in the rate of cell death compared with that of cell proliferation. After feeding, the planarian will grow in size and the cell number will increase again (growth) through a shift in the equilibrium to favour mitosis over cell death. **Regeneration.** Practically any imaginable amputation of the planarian can give rise to two individual animals. Following transverse cuts, the middle fragment forms both an anterior and a posterior blastema. The head, including the eyes and the brain, is regenerated in the anterior blastema and the tail in the posterior blastema. A process of remodelling takes place in the middle fragment, adjusting the organs to the new smaller size of the planarian. When two fragments with different positional values are joined together, intercalary regeneration reforms the missing tissues between the two regions and gives rise to a single planarian. Finally, a sagittal cut induces the formation of a lateral non-pigmented blastema along the length of the planarian body. The missing eye differentiates and the remaining organs are remodelled, giving rise to an entirely regenerated planarian that is smaller than the original animal. **Reproduction.** Some planarian species can alternate between asexual and sexual reproduction depending on factors such as temperature and nutrient availability. Asexual reproduction takes place by fission, usually in the posterior part of the planarian. Sexual reproduction takes place by cross-fertilization between two hermaphroditic planarians producing a polyembryonic egg capsule termed a cocoon. Hatching occurs after 10 days to several weeks depending on the planarian species. **Homeostasis.** A continuous process of renewal of all planarian cells occurs. Modified from Handberg-Thorsager et al., 2008.

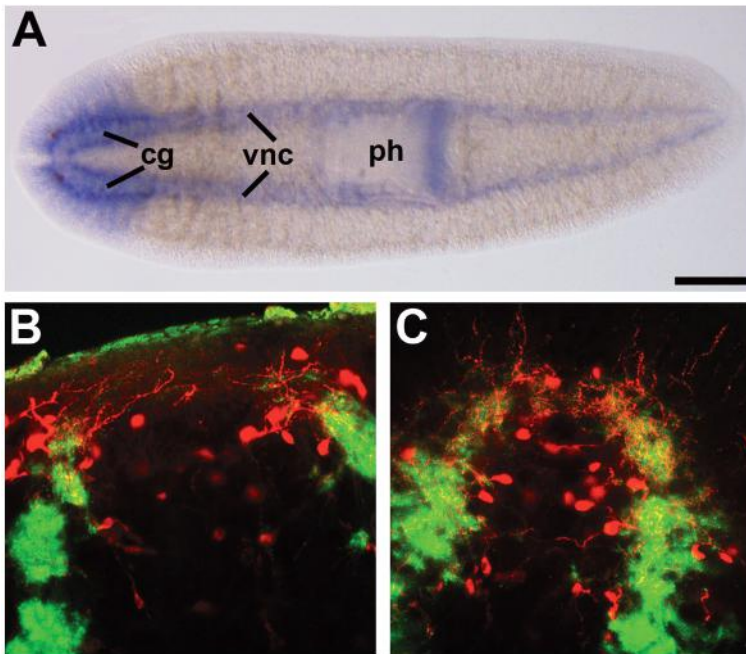




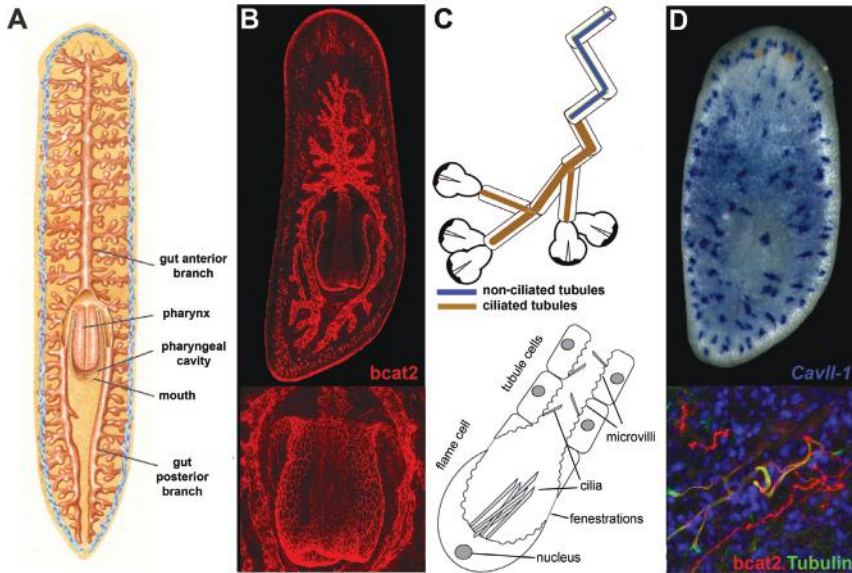
**Figure 3. Neoblasts are totipotent stem cells.** Diagram of the procedure employed to introduce a single neoblast (nb) from non-irradiated asexual donors into the posterior parenchyma of an irradiated sexual host (Wagner et al., 2011). The survivors recover all the regenerative capacities of an asexual planarian.



**Figure 5. Phenotypes generated after silencing BMP and Wnt signalling pathway elements.** (A) Control planarians. (B) *Smed-bmp* and *Smed-Smad1*-silenced planarians show a ventralized phenotype: ectopic differentiation of eyes deep in the mesenchyma and ectopic differentiation of nerve cords in the dorsal region. (C) *Smed-βcat1* and *Smed-WntP-1*-silenced planarians show an anteriorized phenotype. Multiple eyes appear around a large circular cephalic ganglion in *Smed-βcat1*-silenced planarians, and a posterior head appears in *Smed-WntP1*-RNAi animals. (D) Schematic drawing showing abnormal axial patterning after silencing of BMP or Wnt signaling. The antibody used for immunostaining is indicated in each image (anti-synapsin, which labels the CNS, and anti-arrestin, which labels the visual axons). Yellow asterisks indicate the pharynx and yellow arrows indicate the eyes. Red arrows indicate ectopic eyes. Orange arrows indicate duplication of the planarian margins after BMP silencing. D, dorsal view, V, ventral view. Anterior to the left. Modified from Cebrià et al., 2010.



**Figure 6. Structure and regeneration of the planarian CNS.** (A) Whole-mount *in situ* hybridization for *Smed-pc2*, a pro-hormone convertase homologue, expressed in the cephalic ganglia (cg), ventral nerve cords (vnc) and the neural ring in the distal part of the pharynx (ph). (B-C) Regeneration of the planarian CNS detected by double immunostaining with anti-synapsin (in green, panneural) and anti-5HT (in red, serotonergic neurons) after 3days (B) and 7days (C) of regeneration. In (A), anterior is to the left; in (B-C), anterior is to the top. Scale bar in (A), 500  $\mu$ m.

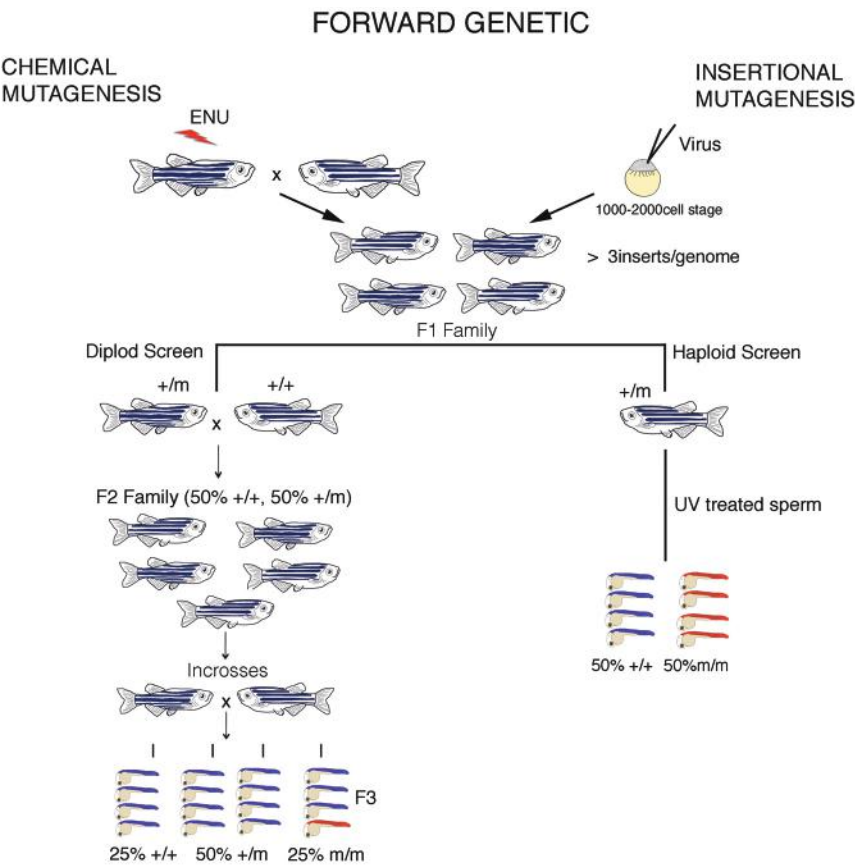


**Figure 8. Planarian digestive and excretory system.** (A) The digestive system consists of a blind diverticulated gut with one anterior and two posterior branches, a single pharynx in the center and a ventrally located mouth. (B) Immunostaining with anti- $\beta$ catenin2, which labels the adherens junctions of epithelial cells. The epithelium of the digestive branches and the different epithelial layers of the pharynx can be observed. (C) The excretory system is composed of long tubular structures, which are highly ciliated in their proximal parts. These connect to a specialized terminal cell, the flame cell, located inside the parenchyma. Adapted from Scimone et al., 2011 and Rink et al., 2011. (D) *In situ* hybridization for carbonic anhydrase (CAVII), which is expressed in the distal, non-ciliated part of the excretory tubules, and immunostaining with anti  $\beta$ catenin2 and anti  $\alpha$ -tubulin, which label the cell junctions of the tubular structures and the cilia, respectively (the nuclei are stained with DAPI, in blue).

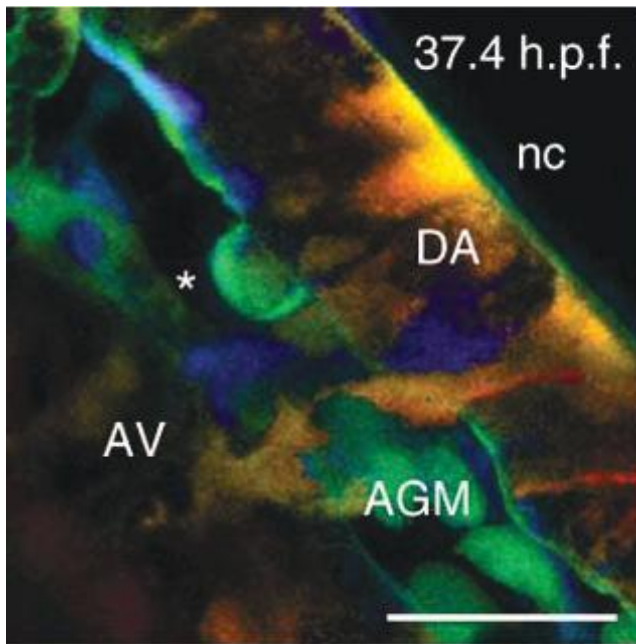
## Chapter 16



**Figure 1. wild-type and transparent mutant zebrafish.** A wild-type zebrafish (top) and a compound roynacre double homozygous mutant, casper (bottom), whose loss of melanocytes and iridophores results in a largely transparent fish uniquely amenable to *in vivo* experimental read outs (adapted from white et al., 2008).



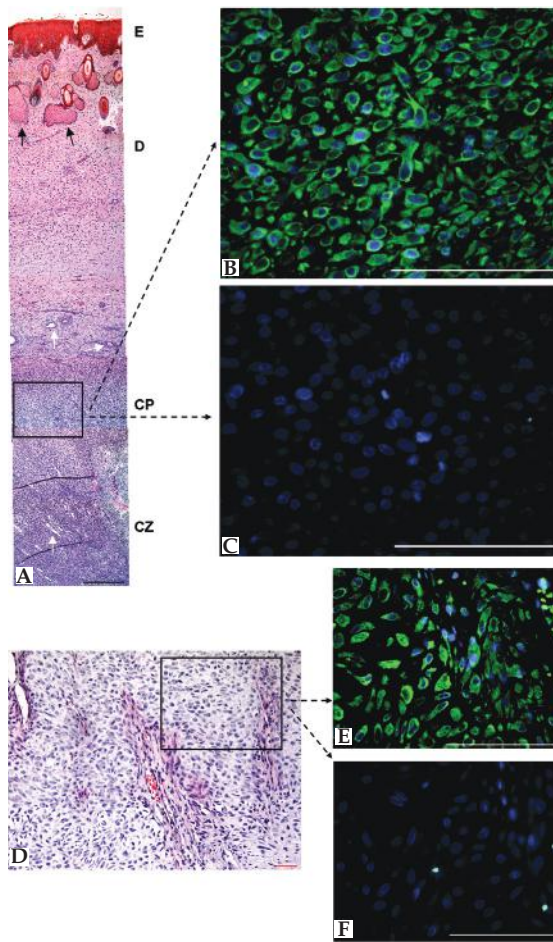
**Figure 2. forward genetic screening.** Diagram depicting chemical and insertional methods for genetic disruption, both resulting in a mutagenized f1 generation whose mutation can be further analyzed by diploid (left) or haploid (right) screening (courtesy of c. santoriello).



**Figure 3. hematopoietic stem/progenitor cells in the vascular niche of the zebrafish embryo.** the caudal hematopoietic tissue (cht) is a vascular plexus in the tail of the embryo between the caudal artery and vein. it is the primary site of definitive hematopoiesis in the embryo, making it comparable to the mammalian fetal liver. shown is the cht of a 48 hour double transgenic embryo soon after it has been colonized by the first stem cells. hematopoietic stem/progenitor cells (green; cd41:egfp) reside in a niche of closely associated endothelial cells (red; flk1:dsred). note: anterior left and dorsal top (courtesy o.tamplin).



## Chapter 17



**Figure 5. Stro-1<sup>+</sup> DaMSCs in the cambial layer of the perichondrium and the cartilaginous zone of an antler.** Paraffin embedded biopsy samples of a velvet antler from a 4 year-old fallow buck (*Dama dama*); samples were taken 46 days after onset of regeneration. **(A)** Cross section of brow tine about 1 cm below the tip, overview, (E) epidermis, (D) dermis, (CP) cambial layer of the perichondrium, (CZ) cartilaginous zone, white arrows = vessels, black arrows = sebaceous glands; HE-staining, scale bar: 500  $\mu$ m. **(B)** STRO-1<sup>+</sup> DaMSCs in the cambial layer of the perichondrium [STRO-1 antibody combined with an anti-mouse IgM secondary antibody conjugated with fluorescence dye (FITC), nuclei counter-stained with Hoechst 33342], scale bar: 100  $\mu$ m. **(C)** Negative control, cambial layer of the perichondrium, same staining as (b) without STRO-1 antibody, scale bar: 100  $\mu$ m, identical exposure times for pictures (b) and (c). **(D)** Cross section of part of a main beam, cartilaginous zone, HE-staining, scale bar: 100  $\mu$ m. **(E)** STRO-1<sup>+</sup> DaMSCs within the cartilaginous zone [same staining as (b)], scale bar: 100  $\mu$ m. **(F)** Negative control, comparable area of the cartilaginous zone, same staining as (e) without STRO-1 antibody, scale bar: 100  $\mu$ m, identical exposure times for pictures (e) and (f). Taken from Rolf et al., 2008.



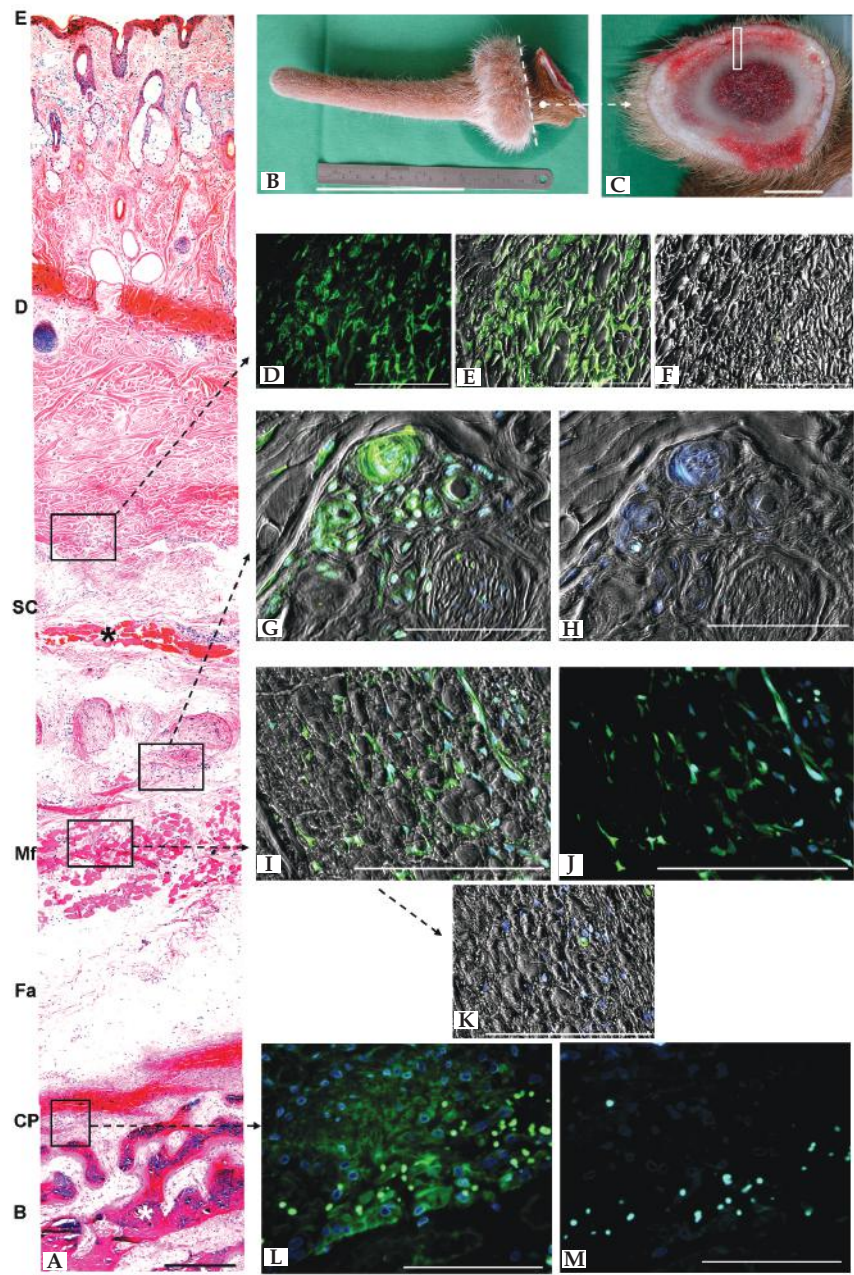
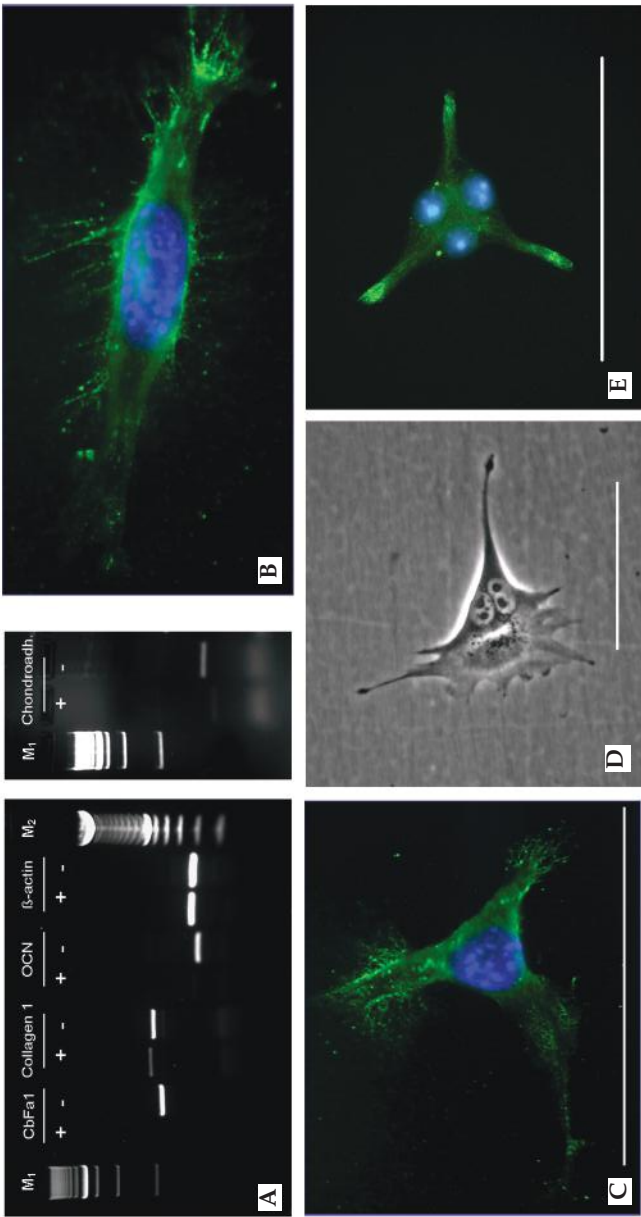
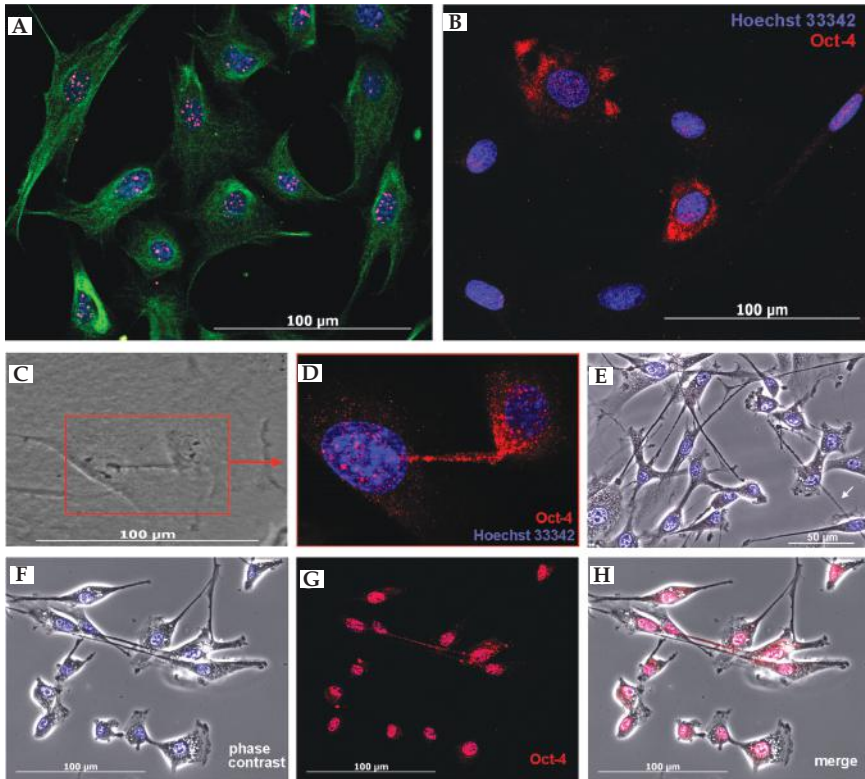


Figure 6. contd....

**Figure 6. Stro-1<sup>+</sup> DaMSCs in different areas of the pedicle.** (A) Methylmetacrylate embedded sample of the pedicle shown in (b) and (c); cross-section, overview, HE-staining. (E) epidermis, (D) dermis, (SC) subcutaneous tissue with superficial muscle (asterisk), (Mf) Part of the frontoscutular muscle, (Fa) fascia, (CP) cambial layer of the periosteum, (B) pedicle bone; white asterisk = bony trabeculae, scale bar: 500  $\mu$ m. (B) Left pedicle and primary velvet antler of a 1 year-old fallow buck (*Dama dama*), the antler was cut below the coronet (dashed line) to obtain a cross-section of the distal pedicle, scale bar: 10 cm. (C) Cross-section of the distal pedicle shown in (b); white rectangle marks the area shown in (a); scale bar: 1 cm. For all pictures (d-m). (D and E) Stro-1<sup>+</sup> DaMSCs within the reticular layer of the dermis, located between thick collagen fibres; (D) Stro-1<sup>+</sup> fluorescence, same area as (e); (e) Fluorescence combined with varel-contrast picture; (F) Negative control; similar area as shown in (e); the small green dots are erythrocytes marked by the fluorescence dyes; identical exposure times for pictures (e) and (f), scale bars: 100  $\mu$ m. (G) Vascular associated Stro-1<sup>+</sup> DaMSCs within the subcutaneous tissue, varel-contrast picture, scale bar: 100  $\mu$ m. (H) Negative control; same area as shown in (g); identical exposure times for pictures (g) and (h), varel-contrast picture, scale bar: 100  $\mu$ m. (I–K) Stro-1<sup>+</sup> DaMSCs between fibres of the frontoscutular muscle, scale bars: 100  $\mu$ m; (I) Fluorescence combined with varel-contrast picture; (J) Stro-1<sup>+</sup> fluorescence only, same area as (i); (K) Negative control, similar area as shown in (i); varel-contrast picture, identical exposure times for pictures (i) and (k); the bright green dots in picture (k) are erythrocytes marked by the fluorescence dyes. (L) Stro-1<sup>+</sup> DaMSCs within the cambial layer of the periosteum; scale bar: 100  $\mu$ m. (M) Negative control, similar area as shown in (l); scale bar: 100  $\mu$ m. Bright dots in pictures (l) and (m) are erythrocytes marked by the fluorescence dyes. Taken from Rolf et al., 2008.

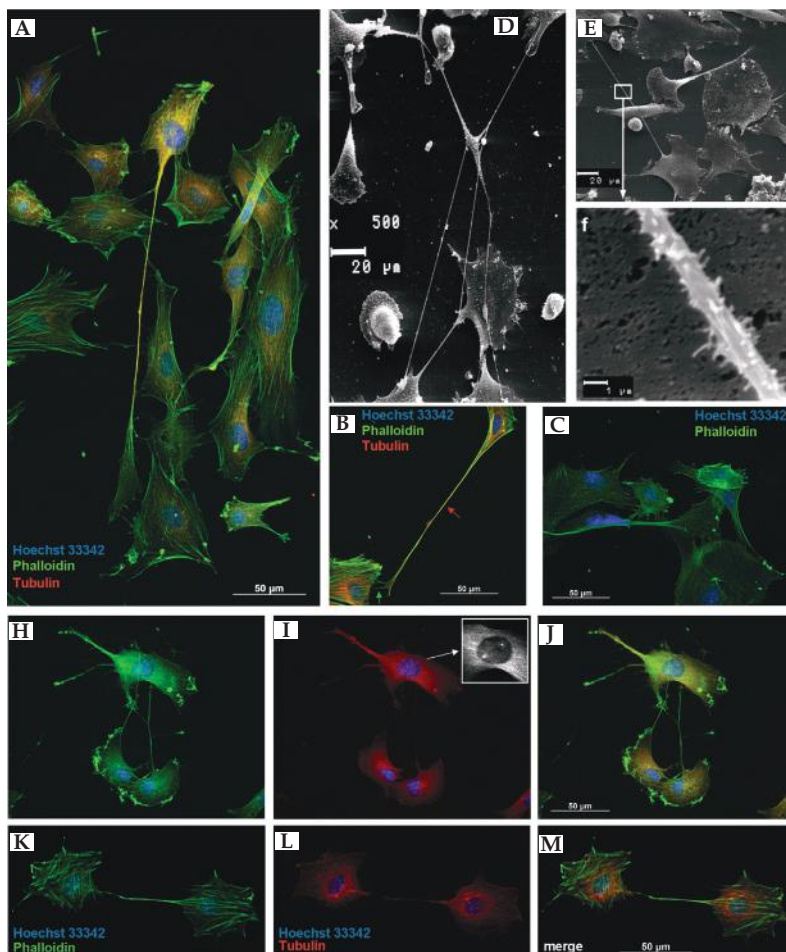


**Figure 7. Expression profiles and morphology of isolated STRO-1<sup>+</sup> DaMSCs.** (A) Expression profiles of Stro-1<sup>+</sup> negative versus Stro-1<sup>+</sup> cells. RT-PCR was used to detect the mRNA of specific markers for the osteogenic [Collagen 1, cbfa1, osteocalcin (OCN)] and the chondrogenic lineages (chondroadherin). Expression of deer β-actin was used for standardization. (+) = Stro-1<sup>+</sup> cells, (-) = Stro-1<sup>-</sup> cells, (M1) = Marker; 500 bp DNA ladder, (M2) = Marker; 100 bp DNA ladder. (B,C) Typical morphology of Stro-1<sup>+</sup> cells isolated from fallow deer antler cell cultures, scale bar: 100 μm. (D,E) Stro-1<sup>+</sup> stem cells with three nuclei, (D) phase contrast picture; (E) same staining as shown in (b) and (c); scale bars: 100 μm. Taken from Rolf et al., 2008.

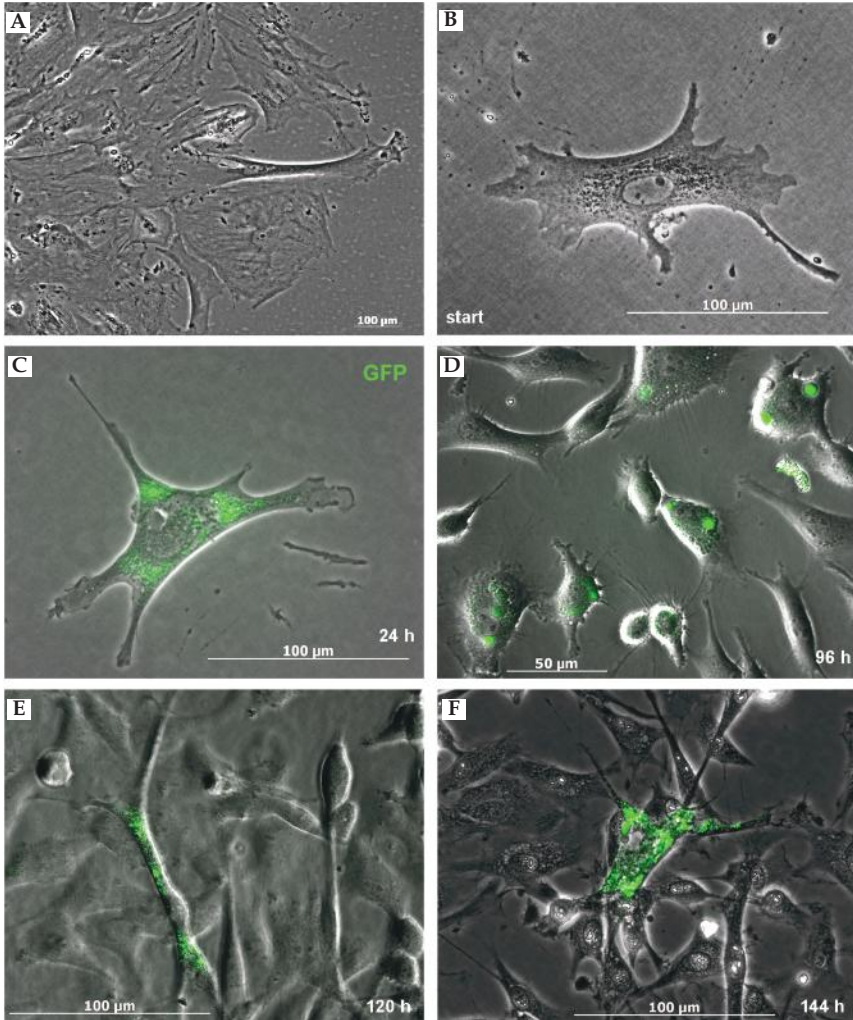


**Figure 8. Intracellular distribution and intercellular transport of Oct4 in Stro-1<sup>+</sup>DaMSC cultures.** (A) DaMSCs 24 hours after cell sorting and subsequent cultivation in stem cell expansion medium. Antibodies against Stro-1 (pseudo-coloured green) and Oct4 (pseudo-coloured red) were used. Nuclei were counter-stained with Hoechst 33342 (blue). Cultured cells showing the surface marker Stro-1 and the transcription factor Oct4 is largely confined to the nuclei. (B) Single-staining with Oct4. Elevated Oct4 expression can be typically observed around 2–4 days after sorting and then about 10% of the cells exhibit intensive Oct4 staining with perinuclear localisation. (C) Phase-contrast picture of two Stro-1<sup>+</sup> cells showing a distinct cell-to-cell connection leading from one cell directly to the adjacent cell. The detail (D) demonstrates the same cells stained with Oct4 antibody (pseudo-coloured red). Oct4 immunostaining is visible perinuclear and within the cell-to-cell connection. One cell exhibits a higher Oct4 concentration which is enriched towards the membranous tube. (E) Phase contrast picture demonstrating that in the majority of cases the intercellular connections are not attached to the substrate. The white arrow point to a floating long-distance cell connection. (f) Phase-contrast picture of Stro-1<sup>+</sup> cells linked by thin cell connections. (G) Oct4 immunostaining (pseudo-coloured red) indicates the presence of Oct4 protein in the nuclei of the cells as well as inside the cell-to-cell connections. (H) Merged image of pictures (f) and (g). Partly taken from Rolf et al., 2012.



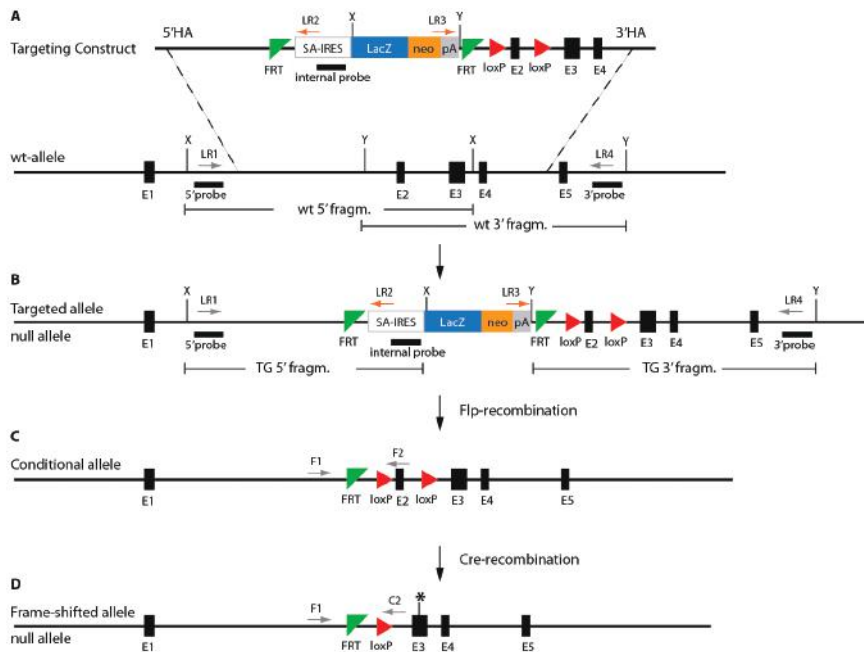


**Figure 9. Cytoskeletal elements of Stro-1<sup>+</sup>DaMSCs and long-distance cell-to-cell connections.** (A,B) Different cell-to-cell connections between STRO-1<sup>+</sup> cells. Intercellular connections are able to bridge long distances even across neighbouring cells (Phalloidin/Tubulin staining, merged images). (B) Thick tubes (diameter >0.4 µm, example is marked by red arrow) contain F-actin and α-tubulin, whereas thin tubes (diameter <0.4 µm, example is marked by green arrow) contain only F-actin. (C) Negative control, staining without Tubulin antibody. (D–F) Scanning electron microscope pictures of a mixed culture of antlerogenic cells. Cells form long connections across neighbouring cells (D,E) and very high magnification (F) proves that the surface of tunneling nanotubes exhibit small appendages; long-distance connections are also possible between morphologically distinguishable cell types (D). (H–M) Multichannel pictures of Phalloidin (H,K) and Tubulin (I,L) stained DaMSCs demonstrate that their intercellular connections continuously consist of F-actin but only partially of microtubules. The visible spindle apparatus (I, detail enlargement) point to an initiating cell division and provides evidence that the used α-tubulin antibody is also efficient in DaMSCs. (J,M). Merged Images. Taken from Rolf et al., 2012.

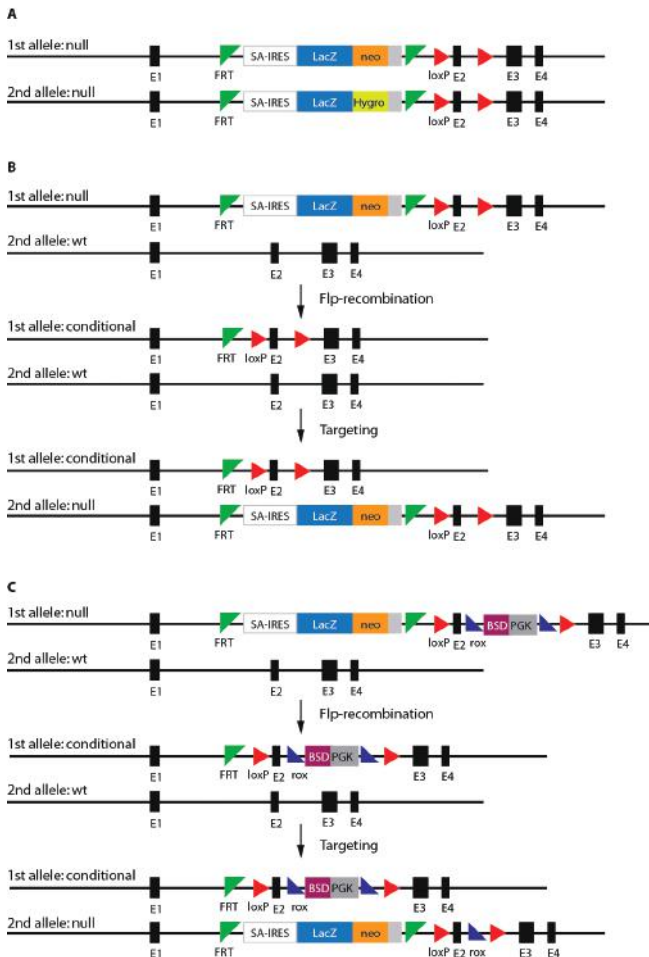


**Figure 10. Mixed culture of Oct4-GFP mouse embryonic fibroblasts cells and Stro-1<sup>+</sup>DaMSCs.** (A) Colony of Oct4-GFP mouse embryonic fibroblasts cells without any GFP expression. (B) Individual Oct4-GFP mouse embryonic fibroblasts cell after 24 hours of pre-cultivation in stem cell expansion medium on the eve of co-cultivation. (C) The same Oct4-GFP mouse embryonic fibroblasts cell after 24 hours of co-cultivation with Stro-1<sup>+</sup> DaMSCs (interacting DaMSCs are outside of the display window). GFP expression (green) is visible within the cytoplasm. (D) Mixed culture after 96 hours of co-cultivation. More GFP<sup>+</sup> cells interacting with GFP2 cells are visible. (E) Cell with distinct GFP expression after 120 hours of co-cultivation. At that time GFP<sup>+</sup> cells interact continuously with GFP2 cells and are well integrated into the forming multilayer. (F) After 144 hours of co-cultivation some cells exhibit widely distributed intracellular GFP expression. (a,b,c,f = phase contrast pictures; d,e = varel contrast pictures; a–e = pictures of living cultures, f = fixed cells). Taken from Rolf et al., 2012.

# Chapter 18

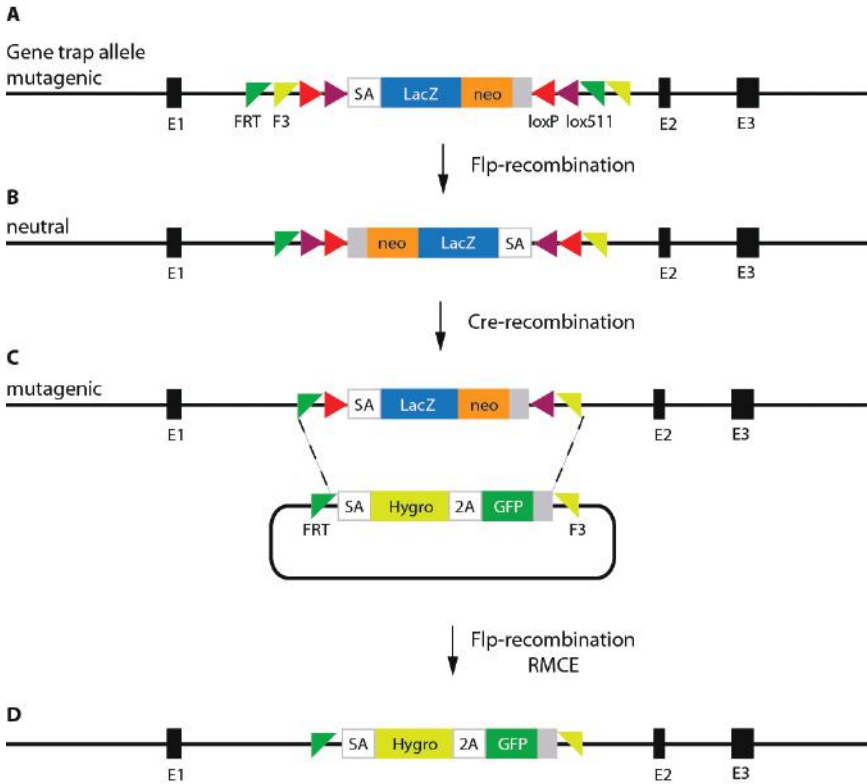


**Figure 2:** Conditional mutagenesis based on the “multi-purpose” allele strategy. **(A)** The targeting construct is composed of a splice-acceptor (SA)-IRES-LacZ-neo fusion followed by a polyadenylation signal (pA) and flanked by FRT sites (green triangles). The frameshifting exon 2 (E2) is flanked by loxP sites (red triangles). **(B)** In the targeted allele (KO first or null allele) transcription starts at exon 1 (E1) and is terminated within the polyA site of the selection cassette. **(C)** The null allele is reverted to a conditional allele by Flp-recombination. **(D)** After excision of the frameshifting exon by Cre-recombination a premature stop codon in exon 3 (E3-asterisk) leads to the activation of the non-sense mediated decay mechanism and degradation of the mRNA. X and Y depict hypothetical restriction endonucleases for Southern –hybridization with the 5’ and 3’ external probes (black horizontal bars). Arrows show primer positions for screening correct targeted events by long-range PCR (LR1, LR2, LR3, LR4), for confirming Flp-recombination (F1, F2) and Cre-recombination (C2).



**Figure 3.** Targeting the second allele. **(A)** If the gene is not essential in ES cells, the second allele can be targeted with the same targeting construct after exchanging the selection marker (from neo to Hygro) using recombinering in bacteria. The cells are selected for resistance to G418 and Hygromycin. **(B)** If the gene is essential in ES cells, then the first targeted allele is reverted to a conditional allele by Flp-recombination. The second allele is targeted with the same targeting construct and a theoretical probability of 50%. **(C)** If the gene is essential in ES cells, the first allele can be targeted by a construct that contains in addition to the SA-IRES-LacZ-neo-pA cassette, a PGK-BSD (blasticidin) cassette positioned downstream of the critical exon and flanked by rox sites (blue triangles) for Dre-recombination. The first targeted allele can be converted to a conditional after Flp-recombination. This configuration resembles the wt allele, assuming that the PGK-BSD cassette, which is inserted in an intron, does not interfere with the expression of the gene. The second allele is targeted with the same targeting construct after removing the PGK-BSD cassette by Dre-recombination in bacteria. The cells are selected for resistance to G418 and blasticidin.





**Figure 4.** Conditional gene trapping based on the Flip-Excision (FIEEx) strategy. **(A)** A gene trap cassette consisting of a splice acceptor (SA)-LacZ-neo fusion and a polyA (grey box) is flanked by a series of site-specific recombination target sites in inverted orientations (FRT, F3, loxP, lox511). The transcript of the gene is captured by the SA and is terminated within the pA site. **(B)** After Flp-recombination and the first inversion, the two F3 sites come into the same orientation and the FRT site that is positioned in-between is deleted. A second inversion by Flp-recombination is not possible because two incompatible sites (F3 and FRT) are flanking now the cassette. In this orientation the cassette cannot capture the transcript of the gene and is neutral. **(C)** The cassette can be inverted once again to the mutagenic orientation by Cre-recombination. **(D)** Recombination mediated cassette exchange (RMCE) can be used to convert the LacZ-neo containing gene-trapped allele to another reporter. In this example Flp mediates recombination between two heterotypic sites and exchanges the trapped cassette to one containing the Hygromycin resistance gene (for selection) and GFP separated by 2A peptide.

## About the Series

*Stem Cells* reviews the current knowledge on stem cell science covering *all* its major topics from basic cell biology to legislation. As opposed to more focused books, *Stem Cells* aims to offer a complete and broad compendium as a key reference for students and more advanced scientists working on any aspect of stem cells. Six sections in two volumes describe: Volume I: i) basic stem cell biology, ii) tissue formation during development, iii) model organisms; Volume II: i) tissue homeostasis and regeneration during adulthood, ii) applications in basic research, medicine and industry, finishing with iii) legislation and ethics; each consisting of self-sufficient and logically connected chapters contributed by prestigious scientists worldwide working on each given topic.

## About the Volume

The first volume of *Stem Cells* deals with the fundamental principles that govern embryonic and somatic stem cell biology. Historically, the identification and characterization of such pathways and general rules of *stemness* occurred during embryonic development and Volume I reflects this with topics spanning cell cycle regulation, epigenetics, and asymmetric cell division in a number of organ systems from planarian to human. Three specific sections discuss i) Basic Stem Cell Biology, ii) Tissue Formation During Development, and iii) Model Organisms with particular emphasis on those more relevant for biomedical research and, thus, leading to the topics addressed in Volume II.

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